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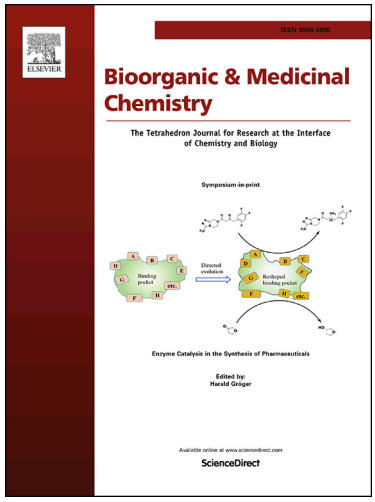

N '-Acyl-*N*-methylphenylenediamine as a Novel Proximity Labeling Agent for Signal Amplification in Immunohistochemistry

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Graphical Abstract

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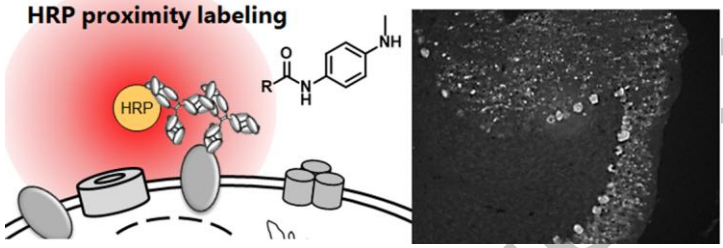

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N'-Acyl-*N*-methylphenylenediamine as a Novel Proximity Labeling Agent for Signal Amplification in Immunohistochemistry

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ABSTRACT

We synthesized novel phenylenediamine derivatives and evaluated them as labeling agents to label proteins in close proximity to a single electron transfer catalyst. We found that *N*'-acyl-*N*-methylphenylenediamine labels tyrosine effectively in a model experiment using tris(bipyridine)ruthenium ($Ru(bpy)_3^{2+}$) as the single electron transfer catalyst. By changing the substituents on the nitrogen atom of the phenylenediamine derivatives, the electrochemical properties of the labeling agent can be drastically changed. On the other hand, horseradish peroxidase (HRP) also catalyzes the reaction with almost the same oxidation potential as $Ru(bpy)_3^{2+}$ (~+1.1 V). HRP proximity labeling is applicable to signal amplification in immunohistochemistry. We evaluated the phenylenediamine derivatives as labeling agents for HRP proximity labeling and signal amplification, and found that *N*'-acyl-*N*-methylphenylenediamine is a novel and efficient agent for signal amplification using HRP in immunohistochemistry.

1. Introduction

Enzyme-linked signal amplification is a key technique to enhance the immunohistochemical detection of protein, mRNA[1], and other biomolecules.[2] This technique enables the high-sensitivity detection of biomolecules, and is useful not only in biochemical research but also in evaluation systems in medicinal research and chemical biology.[3] The signal amplification is based on the covalent bond formation between biomolecules and small reporter molecules in a local environment surrounding enzyme molecules. Although several methods based on the avidin-biotin-peroxidase complex[4,5], enzyme-catalyzed nanoparticle assembly[6], quinone methide generation[7], and ascorbate peroxidase[8] have been reported, the catalyzed reporter deposition (CARD) method using horseradish peroxidase (HRP) is the most widely used. As the covalent labeling agent, reporter-conjugated tyramide, a phenolic compound (Figure 1), has been used. HRP catalyzes the oxidation of tyramide by single electron transfer in the presence of hydrogen peroxide (H_2O_2). The generated tyramide radical reacts with amino acid residues, such as tyrosine, tryptophan, histidine, and cysteine[9], in close proximity to HRP[10]. Most of the signal amplification reactions that involve peroxidase use tyramide as the labeling agent, and therefore, the development of new labeling agent remains important.

HRP is activated by H_2O_2, and heme in the HRP molecule is transformed into a highly reactive species called compound I ($[PPIX]^{\cdot}$ +FeIVO), which can abstract a single electron from a substrate with ~+1.1 V redox potential[11]. On the other hand, we have developed a tyrosine covalent labeling reaction that uses tris(bipyridine)ruthenium ($Ru(bpy)_3^{2+}$) as the single electron transfer catalyst[12,13]. In that method, *N*'-acyl-*N*,*N*-dimethylphenylenediamine labels tyrosine through the activation of ruthenium photocatalyst by visible light irradiation. The redox potential of $Ru(bpy)_3^{2+}$ activated by visible light irradiation ($Ru^{3+} \rightarrow Ru^{2+}$) under the physiological condition is almost the same as that of HRP activated by H_2O_2 (~+1.1 V), and tyrosine residue, a phenolic structure[14], is oxidized (Figure 2). Therefore, we hypothesized that the phenylenediamine derivatives would label amino acid residues, such as a tyrosine residue, even with HRP instead of ruthenium photocatalyst, and would emerge as a novel labeling agent for signal amplification. We found that *N*'-acyl-*N*-methylphenylenediamine is a novel agent for signal amplification using HRP in immunohistochemistry.

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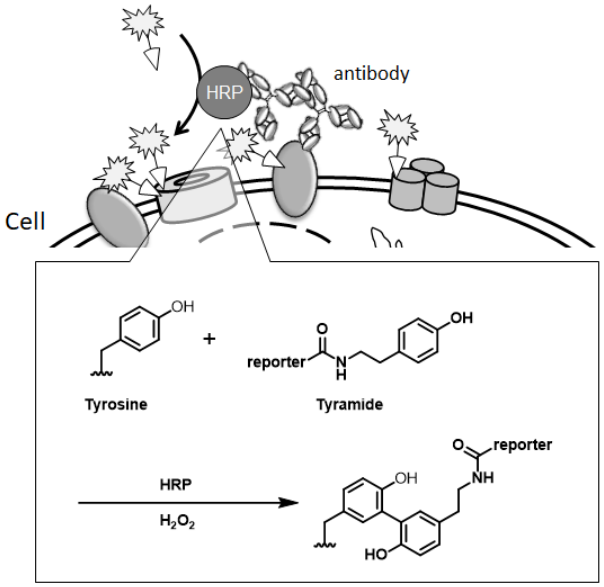


Fig 1. Catalyzed reporter deposition (CARD) using horseradish peroxidase (HRP) and tyramide

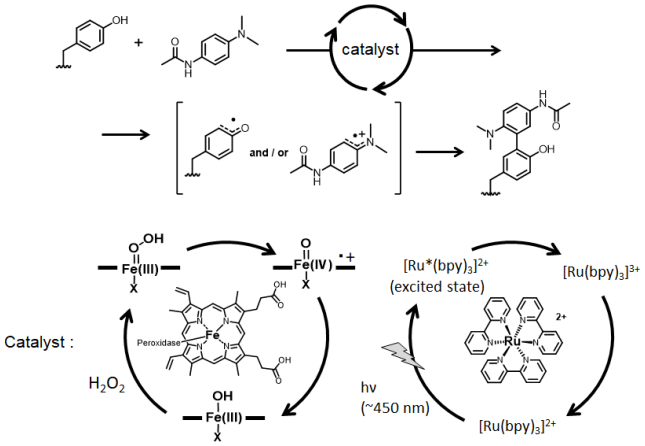
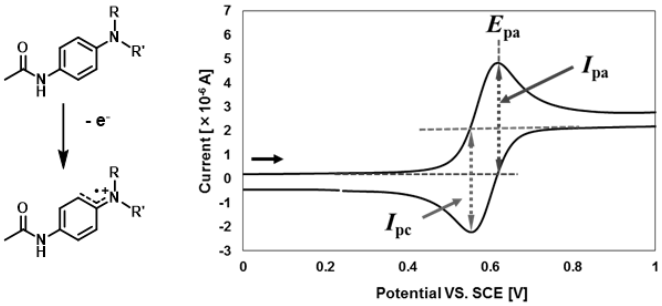


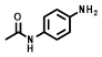
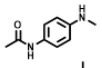
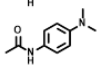
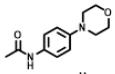
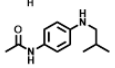
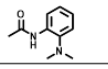
Fig 2. Single electron transfer catalysts, horseradish peroxidase (HRP) and tris(bipyridine)ruthenium ($Ru(bpy)_3^{2+}$)

2. Results and Discussion

We have reported that *N*'-acetyl-*N*,*N*-dimethylphenylenediamine (**3**) is an efficient tyrosine modification agent. As the mechanism of the labeling reaction, not only the pathway via a tyrosyl radical, but also the pathway via a radical species of a labeling agent is suggested (Figure 2). In both pathways, the electrochemical properties that can be oxidized by activated catalysts (~+1.1 V) are essential for efficient labeling agents. The electron density on the phenylenediamine aromatic ring, which is influenced by the substituent on the nitrogen atom, is responsible for the radical properties of the labeling agent. We prepared *N*'-acetyl- phenylenediamine (**1**), *N*'-acetyl-*N*-methylphenylenediamine (**2**), and *N*'-acetyl-*N*,*N*-dimethylphenylenediamine (**3**), and evaluated their electrochemical properties by cyclic voltammetry (Table 1). Increasing the number of substituents on the nitrogen atom decreases E_{pa}, which indicates the potential needed for radical species generation (**1**: 0.71 V, **2**: 0.66 V, **3**: 0.63 V), and increases I_{pc}/I_{pa}, which is an index of the stability of radical species (**1**: 0, **2**: 0.38, **3**: 0.94) (See Supporting Information Figures S1–S7 for details). These results suggest that the radical species of the labeling agent becomes more stable as the number of substituents on the nitrogen atom increases. Morpholino derivative **4** shows a relatively high E_{pa} (0.72 V) and a moderate I_{pc}/I_{pa} (0.63). *N*-isobutyl derivative **5** shows similar properties to *N*-methyl derivative **2** (E_{pa}: 0.65 V, I_{pc}/I_{pa}: 0.31). In the case of ortho-substituted derivative **6**, a high potential (E_{pa}: 1.03 V) is required for oxidation, and the generated radical species is unstable (I_{pc}/I_{pa}: 0).

Table 1. Electrochemical properties of labeling agents.



compound	Structure	E_{pa} [V]	I_{pc}/I_{pa}
1		0.71	0
2		0.66	0.38
3		0.63	0.94
4		0.72	0.63
5		0.65	0.31
6		1.03	0

On the other hand, we previously reported ruthenium-photocatalyst-conjugated tyrosine derivative (**7**) in which $Ru(bpy)_3^{2+}$ is directly conjugated to the amine of L-tyrosine amide, as a model substrate for the evaluation of tyrosine proximity labeling using a single electron transfer catalyst[15]. Using **7** as a substrate, the labeling properties of **1**–**6** were compared. We have reported that **3** labels **7**, and that a covalent bond is formed between the *ortho*-carbon atom of the phenolic oxygen of the tyrosine residue and the *ortho*-carbon atom of the dimethylamino group of **3**[12,15]. Compounds **1** and **2** labeled **7** with comparable efficiency to **3** (Figure 3). The labeling efficiency of compounds **4** and **5** was lower than that of **1** or **2**. Although **6** is a regioisomer of **3**, **6** showed the property of radical species which is significantly different from **3**. Compound **6** did not label **7** (Figure S8). It was suggested that *para*-substituted structure is essential for labeling tyrosine. Interestingly, in the case of labeling with **2**, multi adducts (bis and tris adducts) were observed (Figure 3). As the oxidation reaction and the formation of many types of adducts also proceeded simultaneously,[16] we could not identify the structure of the crosslinked product. The tris adduct might be generated by the covalent dimerization of **2** on the tyrosine residue. The ability of **2** to form multi adducts on a single amino acid residue is considered useful in the reporter deposition technique for signal amplification. As a result of the evaluation using the substrate Ru-$(Pro)_5$-Tyr, which was previously reported by us,[17] the labeling efficiency of **2** was lower than that of **3** (Figure S10). Therefore, **2** is suggested to be a proximity labeling agent with labeling radius shorter than **3**. Comparing the reactivities of **2** and **5**, despite both exhibiting

similar radical characteristics, the reactivity of **5** was low and no multi adducts were detected. This low reactivity might be due to the bulkiness of the *N*-alkyl group.

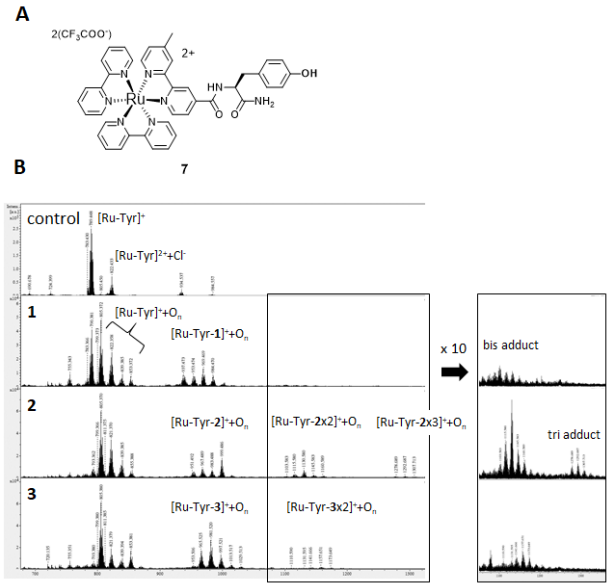


Fig 3. Labeling of **7** using phenylenediamine-type labeling agents. (A) Structure of **7**. (B) Detection of labeled **7** with labeling agents **1**–**3** by mass spectrometry. Left figure: The vertical axis is magnified 10 times in the area of bis and tris adducts.

Based on the results of model experiments using ruthenium photocatalyst, the properties of each labeling agent were evaluated using HRP. As our aim was to apply CARD using HRP, we compared the labeling efficiencies of labeling agents in the model experiment for proximity labeling to antigen-bound HRP. In the model experiment, streptavidin-conjugated HRP (SAv-HRP) was bound to an ELISA plate immobilized with biotin-modified BSA and unmodified BSA (Figure 4A). We labeled proteins on the plate using azide-conjugated labeling agents (**8**–**13**) (Figure 4B). Then, the azide-labeled proteins were functionalized by the click reaction using dibenzocyclooctyne-cyanine 3 (DBCO-Cy3). Labeling efficiencies were evaluated by measuring the fluorescence intensities of wells in the ELISA plate. As a control experiment, the same protocol was carried out with BSA immobilization, but without biotin-modified BSA (Figure 4C, background). In the case of azide-tyramide (**8**) as positive control, a strong signal was observed compared to the control (Figure 4C, signal). It was found that **9**–**11** promoted labeling with HRP as the catalyst although the labeling efficiencies in this model experiment were inferior to that of tyramide **8**. We also evaluated *N*-methyl luminol derivative (**12**), a HRP-mediated tyrosine labeling agent[18,19], and MAUra (**13**), a ruthenium photocatalyst-proximity labeling agent[15]. These labeling agents failed to label the protein in close proximity to HRP. In a solution experiment that used HRP as the catalyst, we found that **12** labeled BSA more efficiently than tyramide[19]. In our model experiment, however, **12** failed to label BSA immobilized on the plate using HRP bound to the solid phase. Considering the previously reported reactivity of MAUra and *N*-methyl luminol derivative,[17] and the result of Figure S10, the effective distance of radical labeling is presumed to be **11**>**10**>**12**,**13**. The labeling radius of the radical species of **12** and **13** might be too small for signal amplification labeling using HRP bound to protein.

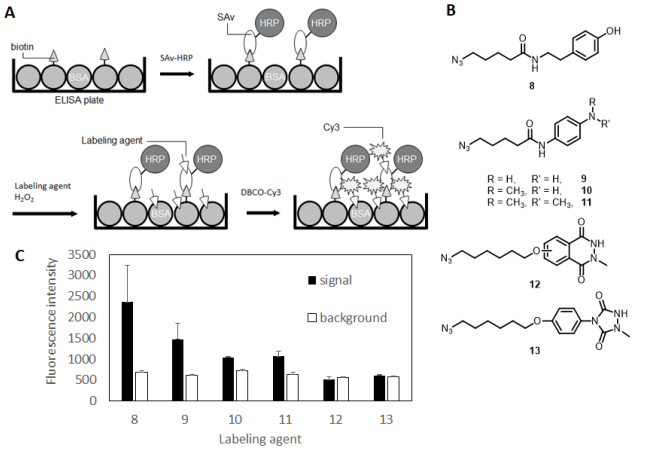


Fig 4. Model experiment using HRP bound to ELISA plate. (A) Assay system of this experiment. (B) Structures of **8**–**13**. (C) Fluorescence intensities of samples treated with labeling agents **8**–**13**.

In the model experiment using BSA as a single kind of substrate, labeling with **9**-**11** was less efficient than tyramide **8**, however, we expected that these labeling agents show high efficiency in experiments using cells. In another model experiment using HRP, proteins on cell membrane surface were used as the substrate. HRP was specifically expressed on plasma membrane. HEK293 cells were transfected with a plasmid for expressing a protein in which HRP is bound to the N-terminus of the transmembrane domain of PDGF receptor (HRP-TM)[9]. Using HRP-TM expressing HEK293 cells, we evaluated **8**–**11**. After labeling, the cell lysate was treated with dibenzocyclooctyne-biotin (DBCO-biotin) to conjugate biotin to the labeled proteins. Labeling efficiencies were evaluated by detecting the biotinylated proteins by western blot analysis using SAv-HRP (Figure 5A). The housekeeping protein, alpha-tubulin, was also detected. Labeling by **8**–**11** was confirmed. Even in the control sample that was not treated with labeling agent, some signals were detected, which were considered to be endogenous biotinylated proteins and proteins nonspecifically labeled with DBCO-biotin via a thiol-yne reaction between the cysteine residues of proteins and DBCO. Compared to the control sample, labeling of various proteins on the cell membrane was detected in all the samples treated with **9**–**11**. In particular, **10** exhibited better labeling efficiency than tyramide derivative **8** (Figure 5B). Although we were unable to identify the labeled proteins, labeling agent **10**, which strongly labeled cellular proteins, was thought to be a suitable candidate for signal amplification using HRP.

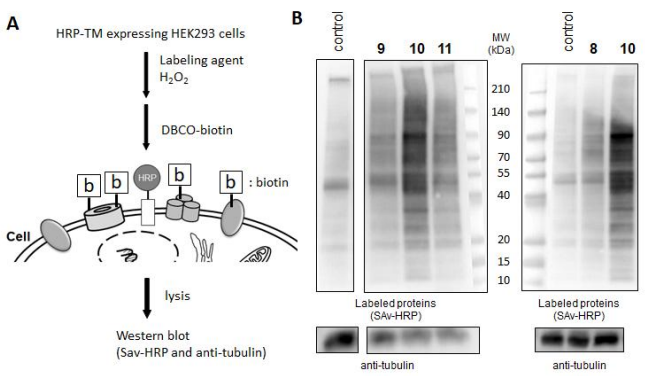


Fig 5. Model experiment using cells expressing HRP on cell membrane. (A) Labeling on cell membrane of HRT-TM expressing HEK293 cells. (B) Western blots of biotin-labeled protein and tubulin.

Finally, we examined whether this method can be applied to experiments using HRP-conjugated secondary antibodies. The signal amplification method is especially useful to confirm the binding of an antibody that cannot produce a sufficient signal in a conventional immunostaining method. Therefore, we performed an experiment where we can amplify a weak signal by signal amplification. We chose calbindin, which is specifically expressed in cerebellar Purkinje cells (PCs), as the target of signal amplification[20,21]. PCs localize in the PC layer found between the molecular layer and the granule cell layer in the cerebellar cortex (Figure 7A), and elaborated dendrites extend into the molecular layer. These localizations are distinctive, and we considered that calbindin on PCs is a suitable target for proof of our strategy because the success of the signal amplification can be easily judged by identifying the labeled area (Figure S11). In previous model experiments (Figures 4 and 5), labeled proteins were visualized by the click reaction between DBCO and azide structures. However, non-specific protein labeling due to the thiol-yne reaction had to be considered as well (Figure S12E)[22]. To reduce noise signals, we considered conjugating biotin to the labeling agent directly. Rhee and co-workers used a desthiobiotin reporter instead of biotin, and accomplished the identification of desthiobiotin-labeled proteins by mass spectrometry[23]. The identification of labeled proteins by mass spectrometry is not adopted in this study. Nevertheless, we recognize the importance of desthiobiotin in future applications. Therefore, we synthesized and used desthiobiotin-conjugated compounds **14**–**17** (Figure 6)[24]. Proteins labeled with desthiobiotin molecules could be visualized by streptavidin-Texas Red. Fluorescence images were acquired with a fluorescence confocal microscope. In the detection of calbindin using a biotin-modified secondary antibody, the signals were very weak, and it was not possible to detect calbindin-positive PCs (Figure 7B). On the other hand, clear fluorescence images were obtained and PCs could be detected after signal amplification by labeling with **14** and **16**. The efficiencies of signal amplification by **14** and **16** were almost the same (Figures 7C and 7D). There was no significant enhancement effect even when **14** and **16** were used at the same time (data not shown). This result suggested that the labeling sites of both agents are the same (mainly tyrosine residues on proteins). A weak signal was observed when the sample was treated with **17**, and almost no signal was detected when the sample was treated with **15** (Figure S12).

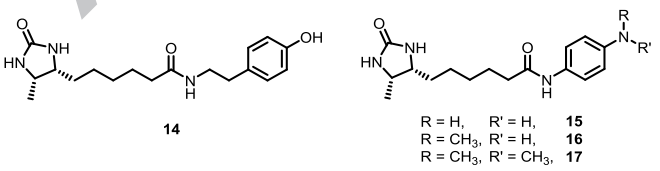


Fig 6. Structures of **14**–**17**.

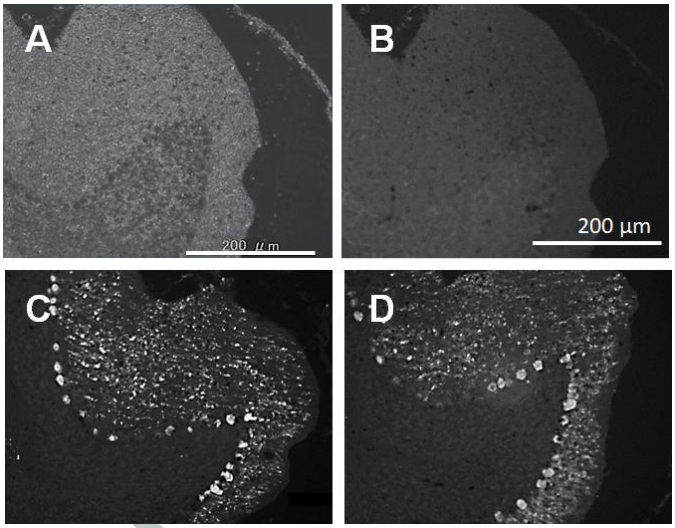


Fig 7. Staining of PCs and signal amplification using anti-calbindin and HRP-conjugated secondary antibodies. (A) Bright-field image of cerebellum. Lower left side is granular layer, upper right side is molecular layer, and between those two layers is the Purkinje cell layer. Exposure time: 1.0 sec. (B) Staining with biotin-conjugated secondary antibody. Exposure time: 0.5 sec. (C) Imaging after signal amplification using tyramide **14**. Exposure time: 0.2 sec. (D) Imaging after signal amplification using **16**. Exposure time: 0.2 sec.

3. Conclusions

We found a novel proximity labeling agent for signal amplification in immunohistochemistry. To identify a novel compound functioning as a proximity labeling agent, we focused on *N*'-acyl-*N*,*N*-dimethylphenylenediamine, which we previously found as a tyrosine labeling agent. We developed the phenylenediamine-type protein labeling agent in this study. Experiments indicated that changing the substituent on the nitrogen atom of *N*'-acetyl-*N*-methylphenylenediamine alters the electrochemical properties of the radical species of the labeling agents. Among the derivatives, *N*'-acyl-*N*-methylphenylenediamine emerged as the most efficient proximity labeling agent for signal amplification using horseradish peroxidase. However, because oxygen addition and multimer formation occurred simultaneously, it was not possible to identify which amino acid residue in the protein is involved in the labeling reactions. The model experiment using a ruthenium-photocatalyst-conjugated tyrosine derivative suggested that *N*'-acyl-*N*-methylphenylenediamine binds to tyrosine residues with oligomerization. This property is required for the deposition of reporter molecules in the proximity of HRP. These results demonstrate that the chemical structures of proximity labeling agents can be modified further for use in signal amplification, and are not limited to tyramide. Research is ongoing to further improve the signal amplification effect by the structural development of labeling agents.

4. Experimental section

4.1. General methods

NMR spectra were recorded on a Bruker biospin AVANCE III (500 MHz for ^{1}H, 125 MHz for ^{13}C) instrument in the indicated solvent. Chemical shifts are reported in units parts per million (ppm) relative to the signal (0.00 ppm) for internal tetramethylsilane for solutions in $CDCl_3$ (7.26 ppm for ^{1}H, 77.0 ppm for ^{13}C) or CD_3OD (3.31 ppm for ^{1}H, 49.0 ppm for ^{13}C). Multiplicities are reported using the following abbreviations: s; singlet, d; doublet, dd; doublet of doublets, t; triplet, q; quartet, quint.; quintet m; multiplet, br; broad, *J*; coupling constants in Hertz. IR spectra were recorded on a JASCO FT/IR-4100 spectrometer. IR spectrum was recorded on a JASCO Corporation FT/IR-4100 FT-IR Spectrometer. ATR PRO ONE was attached to the FT/IR-4100 in measuring solid IR spectroscopy by single reflection attenuated total reflection. Only the strongest and/or structurally important peaks were reported as the IR data given in cm□−1. The absorption spectra were measured with JASCO V-670. HRMS (ESI-TOF-MS) were measured with Bruker micrOTOF II. Only the strongest and/or structurally important peaks are reported as IR data given in cm^{-1}. High-resolution mass spectra (HRMS) were recorded on a Bruker ESI-TOF-MS (micrOTOF II). Analytical thin layer chromatography (TLC) was performed on a glass plate of silica gel 60 GF254 (Merck). Silica gel (Fuji Silysia, CHROMATOREX PSQ 60B, 50-200 μm) was used for column chromatography. Most commercially supplied chemicals were used without further purification. Compound **1** was purchased from TCI. Compounds **3**[12], **7**[15], **11**[12], **12**[18], **13**[15] were prepared according to the previously reported procedures, and the observed spectra were well consisted to the reported spectra.

*4.2. N'-acetyl-N-methylphenylenediamine (**2**)*

A solution of 4'-aminoacetanilide (200 mg, 1.33 mmol), methyl iodide (66.1 μL, 1.07 mmol) and potassium iodide (221 mg, 1.60 mmol) in 8.0 mL acetonitrile was heated at 90 °C for 6 h, and then was cooled to room temperature and filtered. The filtrate was concentrated and purified by silica gel column chromatography (CH_2Cl_2 : methanol = 15 : 1) to afford a solid product (39.3 mg, 18%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (d, J = 8.8 Hz, 2H), 7.13 (br, 1H), 6.56 (d, J = 8.8 Hz, 2H), 2.81 (s, 3H), 2.12 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.3, 146.7, 128.2, 122.6, 112.7, 31.2, 24.4; FT-IR (neat) 3296, 3065, 2882, 2812, 1655, 1618, 1520, 1511 cm^{-1}; Mp 83 °C ; HRMS (ESI, positive): calcd for $[C_9H_{12}N_2O+Na]^+$: 187.0842, found 187.0842.

*4.3. N-(4-morpholinophenyl)acetamide (**4**).*

A solution of 4'-aminoacetanilide (100 mg, 0.665 mmol), bis(bromoethyl)ether (635 μL, 0.505 mmol), potassium iodide (188 mg, 1.13 mmol) and potassium carbonate (156 mg, 1.13 mmol) in 15.0 mL acetonitrile was heated at 90 °C for 24 h, and then was cooled to room temperature and filtered. The filtrate was concentrated and purified by silica gel column chromatography (CH_2Cl_2 : methanol = 15: 1) to afford a solid product (130.4 mg, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 (d, J = 8.8 Hz, 2H), 7.26 (br, 1H), 6.87 (d, J = 8.8 Hz, 2H), 3.85 (t, J = 4.8 Hz, 4H), 3.11 (t, J = 4.8 Hz, 4H), 2.14 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.3, 148.4, 130.8, 121.7, 116.4, 67.0, 47.9, 24.5. ; FT-IR (neat) 3276, 3190, 3119, 2958, 2848, 2826, 1654, 1533, 1456, 1119 cm^{-1}; Mp 208 °C ; HRMS (ESI, positive): calcd. for $[C_{12}H_{16}N_2O_2+H]^+$: 221.1285, found 221.1286

4.4. N-*[4-[(2-methylpropyl)amino]phenyl]acetamide (**5**).*

This compound was prepared according to the similar procedure with **2**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (br, 1H), 7.24 (d, *J* = 8.8 Hz, 2H), 6.51 (d, *J* = 8.8 Hz, 2H), 3.68 (br, 1H), 2.87 (d, J = 6.8 Hz, 2H), 2.06 (s, 3H), 1.87-1.80 (m, 2H), 0.95 (d, *J* = 6.8 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.7, 145.7, 128.1, 122.5, 112.7, 52.1, 28.0, 24.1, 20.5; FT-IR (neat) 3296, 3139, 3068, 2955, 2928, 2869, 1656, 1519, 1473, 1314, 1262 cm^{-1}; Mp 78-80 °C; HRMS (ESI, positive): calcd. for $[C_{12}H_{18}N_2O+Na]^+$: 229.1311, found 229.1311.

*4.5. N-(2-(dimethylamino)phenyl)acetamide (**6**)*

To a solution of 3'-aminoacetanilide (500 mg, 3.33 mmol) in 4.00 mL THF was added formaldehyde (5.16 mL). The reaction mixture was cooled to 0 ºC and stirred for 5 min. Sodium borohydride (629 mg, 16.7 mmol) was then added slowly to the reaction mixture and the reaction was stirred at 0 ºC for 20 min. H_2O (5.16 mL) was added and the pH was adjusted to pH 7 using $NaHSO_4$. The product was extracted into AcOEt. The organic layers were combined and the solvent was removed *in vacuo*. purified by silica gel column chromatography (Toluene : Acetone = 4 : 1) afforded (404 mg, 68%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.45 (s, 1H), 8.33 (d, *J* = 8.0, 1H), 7.16 (d, *J* = 8.0 Hz, 1H), 7.11 (t, *J* = 2.0 Hz, 1H), 7.04 (t, *J* = 4.0 Hz, 1H), 2.64 (s, 6H), 2.22 (s, 3H).

*4.6. Azide-conjugated tyramide (**8**)*

To a solution of tyramine (141.9 mg, 1.03 mmol), 5-azidopentanoic acid (133.4 mg, 0.93 mmol), DMAP (184.1 mg, 1.51 mmol) and DIEA (0.26 mL, 1.51 mmol) in 7.0 mL of DMF was added EDCI-HCl (214.2 mg, 1.12 mmol) at room temperature. After stirring at room temperature for 27 h, AcOEt and H_2O were added to the reaction mixture. The organic layer was washed by aqueous HCl solution (1 M) and saturated aqueous NaCl solution, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by silica gel chromatography (Hexane : AcOEt = 1 : 4) to give **8** (162.9 mg, 67%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.04 (d, *J* = 8.4 Hz, 2H), 6.79 (d, *J* = 8.4 Hz, 2H), 5.46 (br, 1H), 3.50 (quint, *J* = 6.8 Hz, 2H), 3.26 (t, *J* = 6.8 Hz, 2H), 2.74 (t, *J* = 6.8 Hz, 2H), 2.16 (t, *J* = 7.2 Hz, 2H), 1.72-1.65 (m, 2H) 1.61-1.58 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.4, 155.3, 129.7, 115.7, 51.0, 41.0, 35.9, 34.6, 28.2, 22.9; FT-IR (neat) 3298, 3019, 2937, 2869, 2691, 2607, 2502, 2097, 1645, 1614, 1594, 1544, 1515, 1453, 1362, 1242, 1172, 1106, 1044 cm^{-1}; HRMS (ESI, positive): *m/z* calcd. For $C_{13}H_{18}N_4O_2$ $[M+Na]^+$: 285.1322, found 285.1320.

*4.7. 5-Azido-N-(4-aminophenyl)pentanamide (**9**)*

Isobutyl chloroformate (640 μL, 8.9 mmol) and *N*-methylmorpholine (833 μL, 13.9 mmol) were added dropwise to a solution of 5-azidopentanoic acid (1202 mg, 8.4 mmol) in THF (20 mL) at 0 °C. The resulting solution was stirred at 0 °C. After 1.5 h, a solution of *N*-(*tert*-Butoxycarbonyl)-1,4-phenylenediamine (685 mg, 3.3 mmol) in THF (1 mL) was added and the reaction mixture was stirred at room temperature for 12 h. The reaction mixture was quenched with aqueous HCl solution (1 M) to acidify and washed with AcOEt. A solution of aqueous NaOH solution (6 M) was added to the mixture to become basic. The product was extracted with AcOEt and the solvent was removed under reduced pressure. Purification by silica gel column chromatography (AcOEt: hexane = 1:1) afforded intermediate (Boc derivative)**.** Then, to a solution of intermediate (907 mg, 27.2 mmol) in CH_2Cl_2 (40 mL) was added TFA (4 mL, 52.2 mmol) at room temperature for 2.5 h. The reaction mixture was then diluted with saturated aqueous $NaHCO_3$ solution and extracted with CH_2Cl_2 and dried over $MgSO_4$.The CH_2Cl_2 solvent was removed under reduced pressure. Purification by silica gel column chromatography (AcOEt: hexane = 1:1) afforded compound **9** (2 steps, 503 mg, 25%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (s, 1H), 7.23 (d, *J* = 9.0

Hz, 2H), 6.60 (d, *J* = 8.5 Hz, 1H), 3.42 (br 2H), 3.27 (t, *J* = 6.8 Hz, 2H), 2.29 (t, *J* = 7.5, 2H), 1.77-1.71 (m, 2H), 1.65-1.59 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8, 143.5, 129.2, 122.3, 115.4, 51.2, 36.6, 28.4, 22.9; FT-IR (neat) 3418, 3320, 3049, 2950, 2870, 2103, 1652, 1534, 1468 cm^{-1}; Mp 73 °C; HRMS (ESI, positive) : calcd. for $[C_{11}H_{15}N_5O+H]^+$: 234.1349, found 234.1349.

*4.8. 5-Azido-N-(4-(methylamino)phenyl)pentanamide (**10**)*

A mixture of **9** (50 mg, 0.214 mmol), methyl iodide (60.6 mg, 0.428 mmol) and potassium carbonate (50.3 mg, 0.364 mmol) in 5.0 mL acetonitrile was heated at 90 °C for 6 h, then was cooled to room temperature and filtered. The filtrate was concentrated and purified by column chromatography on silica gel (AcOEt : hexane = 1: 1) to afford a solid product (8.0 mg, 15%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.29 (d, *J* = 9.0 Hz, 2H), 7.06 (br, 1H), 6.58 (d, *J* = 8.0 Hz, 2H), 3.32 (t, *J* = 6.8 Hz, 2H), 2.82 (s, 3H), 2.34 (t, *J* = 7.3, 2H), 1.84-1.78 (m, 2H), 1.71-1.65 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.4, 146.5, 128.3, 122.3, 112.9, 51.4, 36.8, 31.3, 28.5, 23.0; FT-IR (neat) 3292, 2929, 2871, 2818, 2096, 1653, 1520, 1457, 1466 cm^{-1}; Mp 68-71 °C; HRMS (ESI, positive): calcd. for $[C_{12}H_{17}N_5O+H]^+$: 248.1506, found 249.1506.

*4.9. Desthiobiotin-conjugated tyramide (**14**).*

To a solution of D-desthiobiotin (28.9 mg, 0.135 mmol), tyramine (21.2 mg, 0.162 mmol) and HOBt・H_2O (24.8 mg, 0.162 mmol) were added EDCI-HCl (31.1 mg, 0.162 mmol) at room temperature. After stirring at room temperature for 32 h, the reaction mixture was concentrated *in vacuo*. The residue was purified by silica gel chromatography (CH_2Cl_2 : MeOH = 10 : 1 → 7 : 1) to give **14** (20.1 mg, 47%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) d 7.02 (d, *J* = 8.4 Hz, 2H), 6.92 (br, 1H), 6.70 (d, *J* = 8.4 Hz, 2H), 3.80 (q, *J* = 7.2 Hz, 1H), 3.69 (quint, *J* = 7.2 Hz, 2H), 3.37-3.34 (m, 2H), 2.69 (t, *J* = 6.8 Hz, 2H), 2.15 (t, *J* = 7.2 Hz, 2H), 1.60-1.56 (m, 2H), 1.48-1.44 (m, 2H) 1.34-1.25 (m, 4H), 1.10 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD) d 176.1, 166.2, 156.9, 139.1, 131.2, 130.7, 129.6, 126.1, 116.2, 57.4, 52.7, 42.1, 37.0, 35.6, 35.4, 30.9, 30.7, 30.1, 27.1, 26.8, 21.3, 15.6; FT-IR (neat) 3639, 3243, 3085, 2921, 2854, 1685, 1636, 1556, 1542, 1514, 1431, 1360, 1229, 1149, 1103, 1026 cm^{-1}; Mp 64-67 °C; HRMS (ESI, positive): *m/z* calcd. for $C_{18}H_{27}N_3O_3$ $[M+Na]^+$: 356.1945, found 356.1950..

*4.10. N'-Desthiobiotin-phenylenediamine (**15**)*

Isobutyl chloroformate (364 µL, 2.8 mmol) and 4-methylmorpholine (308 µL, 2.8 mmol) were added to a solution of D-desthiobiotin (501.6 mg, 2.3 mmol) in DMF (10 mL). This solution was stirred at room temperature for 1.5 hour. Then, *N*-Boc-*p*-phenylendiamine (585.5 mg, 2.8 mmol) was added in resultant mixture and it was stirred at room temperature overnight. The solvent was removed in vacuo and the residue was purified by column chromatography on silica gel with CH_2Cl_2 : CH_3OH (10 : 1). Next, the resultant mixture was dissolved in CH_2Cl_2 (2.7 mL) and TFA (0.3 mL, 10%) was added. It was stirred at room temperature for 1.5 hour. The solvent was removed in vacuo to give compound **15** (9.5 mg, 3 steps 1 %) as a yellow solid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.74 (d, *J* = 8.9 Hz, 2H), 7.33 (d, *J* = 8.9 Hz, 2H), 3.86-3.79 (m, 1H), 3.73-3.68 (m, 1H), 3.06 (s, 1H), 2.93 (s, 1H), 2.40 (t, *J* = 7.4 Hz, 2H), 1.73 (quint, *J* = 7.2 Hz, 2H), 1.51-1.40 (m, 6H), 1.11 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.9, 129.0, 128.2, 125.3, 56.3, 51.7, 35.6, 33.0, 29.2, 29.0, 26.1, 24.7, 21.4, 15.4; FT-IR (neat) 3269, 3060, 2925, 2850, 2606, 1663, 1542, 1512, 1457, 1420, 1378, 1310, 1257, 1177, 1132 cm^{-1}; Mp 84-86 °C; HRMS (ESI, positive): *m/z* calcd. for $C_{16}H_{24}N_4O_2$ $[M+Na]^+$: 327.1791, found 327.1796.

*4.11. N'-Desthiobiotin-N-methylphenylenediamine (**16**)*

To a solution of *N*-Boc-*p*-phenylendiamine (53.7 mg, 0.258 mmol) in MeOH (5 mL), paraformaldehyde (37.4 mg) and sodium methoxide (67.4mg, 1.248 mmol). The mixture was stirred for 11 h at reflux under an inert atmosphere. Then, the reaction mixture was reacted with $NaBH_4$ (14.5 mg, 0.383 mmol) at room temperature and refluxed for an additional 4 h. The mixture was concentrated *in vacuo*. The residue was dissolved in saturated aqueous NH_4Cl, was extracted with ethyl acetate and dried over Na_2SO_4. The solvent was concentrated *in vacuo* and was purified by column chromatography on silica gel with Hexane : ethyl acetate (7 : 3) to give *N*'-Boc-*N*-methyl-*p*-phenylendiamine as a yellow oil (45.7 mg, 80%). *N*'-Boc-*N*-methyl-*p*-phenylendiamine was dissolved in 4 M HCl/dioxane (500 µL) and stirred at room temperature for 2 hours. The solvent was concentrated *in vacuo* and was dissolved in DMF (1 mL). This solution was added D-desthiobiotin (45.1 mg, 0.210 mmol), EDCI-HCl (41.1 mg, 0.214 mmol), HOBt (28.2 mg, 0.209 mmol) and DIEA (103.4 µL, 0.6 mmol). The mixture was stirred at room temperature for 15 h and was concentrated *in vacuo*. The residue was dissolved in saturated aqueous $NaHCO_3$, was extracted with ethyl acetate and dried over Na_2SO_4. Then, the residue was purified by preparative thin layer chromatography with CH_2Cl_2 : MeOH = 10 : 1 to give **16** as a white solid (15.1 mg, 24%). ^{1}H NMR (500 MHz, CD_3OD) δ 7.29 (d, *J* = 8.9 Hz, 2H), 6.61 (d, *J* = 8.9 Hz, 2H), 3.86-3.80 (m, 1H), 3.74-3.70 (m, 1H), 3.37 (s, 1H), 2.76 (s, 3H), 2.35 (t, *J* = 7.5 Hz, 2H), 1.73 (quint, *J* = 7.4 Hz, 2H), 1.56-1.34 (m, 6H), 1.12 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 172.8, 164.8, 147.1, 128.2, 121.9, 112.3, 56.0, 51.3, 36.2, 29.7, 28.3, 28.9, 25.8, 25.5, 14.2; FT-IR (neat) 3242, 3140, 3067, 2925, 2855, 1691, 1652, 1602, 1517, 1430, 1401, 1376, 1347, 1306, 1249, 1176, 1155, 1101, 1062 cm^{-1}; Mp 85-87 °C ; HRMS (ESI, positive): *m/z* calcd. for $C_{17}H_{26}N_4O_2$ $[M+Na]^+$: 341.1948, found 341.1945.

*4.12. N'-Desthiobiotin-N,N-dimethylphenylenediamine (**17**)*

A solution of *N*, *N*-dimethyl-*p*-phenylenediamine in DMF (500µL) was added D-desthiobiotin (23.6 mg, 0.110 mmol), EDCI-HCl (20.5 mg, 0.106 mmol), HOBt (14.3 mg, 0.106 mmol) and DIEA (26.0 µL, 0.100 mmol). The mixture was stirred at room temperature for 22 h and was concentrated *in vacuo*. The residue was dissolved in H_2O, extracted with ethyl acetate and dried over Na_2SO_4. The solvent was concentrated *in vacuo* and was purified by column chromatography on silica gel with CH_2Cl_2 : MeOH (10 : 1) to give **17** as a black solid (8.8 mg, 24%). ^{1}H NMR (500 MHz, CD_3OD) δ 7.81 (s, 1H), 7.39 (d, *J* = 9.0 Hz, 2H), 6.69 (d, *J* = 9.0 Hz, 2H), 5.80 (s, 1H), 4.69 (s, 1H), 3.85-3.80 (m, 1H), 3.71-3.68 (m, 1H), 2.90 (s, 6H), 2.33 (t, *J* = 7.3 Hz, 2H), 1.74 (quint, *J*=6.9 Hz, 2H), 1.53-1.21 (m, 6H), 1.10 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.1, 163.8, 147.9, 128.2, 121.7, 113.1, 56.0, 51.4, 41.0, 36.7, 29.5, 28.4, 25.7, 25.3, 15.8; FT-IR (neat) 3231, 3109, 3057, 2929, 2852, 2789, 1699, 1653, 1596, 1518, 1457, 1419, 1375, 1340, 1315, 1253, 1161, 1129, 1103, 1057 cm^{-1}; Mp 138-139 °C ; HRMS (ESI, positive): *m/z* calcd. for $C_{18}H_{28}N_4O_2$ $[M+Na]^+$: 355.2104, found 355.2103.

4.13. Electrochemical measurement.

Electrochemical measurements were made with a Hokuto-Denko HZ-5000 analyzer (observed in CH_3CN; [compound] = ca. 1 mM; $[NBu_4PF_6]$ = 0.1 M; working electrode: Pt, counter electrode: Pt, reference electrode: Ag/AgCl; scan rates were 100 mV/s). After the measurement, ferrocene (Cp_2Fe) was added to the mixture and the potentials were calibrated with respect to the Cp_2Fe/Cp_2Fe^+ redox couple[25].

*4.14. Evaluation of labeling efficiency using **7***

To a solution of **7** (final concentration 10 μM) in MES buffer (10 mM, pH 6.0), labeling reagent (a 100 mM stock solution in DMSO, final concentration 1 mM) were added, and the mixture was briefly vortexed and incubated at room temperature for 5 min. The solution was vortexed and the reaction was performed with the irradiation of the light (RELYON, Twin LED light, 455 nm) on ice 0.5 cm from the light source for 5 min irradiated with light on ice for 5 min. The reaction mixture (0.5 μL) with 0.1% TFA 0.5 μL was mixed with 0.5 μL of CHCA solution (0.5 mg/mL solution in acetonitrile : 0.1% TFA = 1 : 1) on MALDI-TOF plate and dried at room temperature. The modified protein peaks were detected by MALDI-TOF MS (Bruker, UltrafleXtreme).

4.15. Evaluation of labeling efficiency using HRP bound to ELISA plate.

A solution of maleimide-biotin (Aldrich) in DMSO (100 μM) was added to a solution of BSA (10 μM) in 1×PBS, then incubated at room temperature for 1 h. After removing excess amount of maleimide-biotin by Sephadex G-25 column (GE), biotin-conjugated BSA was obtained. A solution of biotin-BSA (1 μg/mL in 1×PBS) 100 μL was added to each well on 96 well ELISA plate (flat-bottom, black, high binding) and incubated at 4 °C for 12 h, then the ELISA plate was washed three times using 1×PBS 200 μL. A solution of BSA (3% (w/v) in 1×PBS) 200 μL was added to each same well on ELISA plate and incubated at 37 °C for 4 h, then the ELISA plate was washed three times using 1×PBS 200 μL. A solution of SAv-HRP (final conc. 1 μg/mL in 1×PBS) 100 μL was added to each same well on ELISA plate and incubated at room temperature for 1 h, then the ELISA plate was washed three times using 1×TTBS 200 μL. A solution of azide-conjugated labeling agents (4 μM) and H_2O_2 (1 mM) in 1×TBS 100 μL was added to each well on ELISA plate. The ELISA plate incubated at room temperature for 10 min, then the ELISA plate was washed one time using 1×TTBS 200 μL. A solution of DBCO-Cy3 (Aldrich, 10 mM in DMF, final conc. 4 μM in 1×PBS) 100 μL was added to each well on ELISA plate and incubated at 37 °C for 30 min, then the ELISA plate was washed three times using 1×TTBS 200 μL. Fluorescence intensity of each well on ELISA plate was measured by microplate reader (Infinite F200).

4.16. Evaluation of labeling efficiency using cells expressing HRP on cell membrane.

HEK293 cells (2 x 10^5 cells/well) were seeded on 24 well plate in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FBS at 37 °C in an atmosphere of 5% CO_2 and 100% humidity for 24 h. Transfections were performed using Lipofectamine 2000 (Thermo Fisher Scientific, 2.5 μL/well) and pCAG HRP-TM[9] (500 ng/well, the plasmid was donated by Prof. Hyun-Woo Rhee), and cells were cultured for 24 h. Cells were treated with each compound (final 500 μM in medium) for 30 min at 37ºC. H_2O_2 (final 1 mM) is added to each well. After 1 min incubation, quencher (the mixture of 50 mM Trolox, 100 mM Ascorbate and 100 mM NaN_3) was added. Medium was removed and cells were lysed in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX). Lysate was treated with DBCO-biotin (Aldrich final 100 μM) for 1 h after the size exclusion column using Micro Bio-Spin 6 column (Bio Rad). The proteins were separated by SDS-PAGE with 4-20% acrylamide gels (Bio Rad) and were transferred to PVDF membrane. It was incubated with Immuno Block (DS Pharma) for 1 h. and washed by TTBS buffer for 15 min in 3 times. HRP-streptavidin was treated at 4 ºC for 16 h. the membrane was treated with Immobilon® Forte Western Substrates (Millipore). The chemiluminescence images were obtained with Fusion Solo 4S (Vilber Lourmat).

4.17. Staining of PC and signal amplification using anti-calbindin and HRP-conjugated secondary antibody

The tissue sections of mouse brain were deparaffinized by washing these sections in lemosol for 5 minutes, three times, and in EtOH for 5 minutes, three times. Then, these sections were washed sequentially with 90% EtOH, 80% EtOH, 70% EtOH, PBS. These were washed again in PBS twice after treated with 3% H_2O_2/MeOH for 30 minutes. Sections were treated with 5% BSA/PBS and were washed by TTBS three times. The sections were treated with anti-calbindin rabbit IgG antibody (Cell Signaling Technology, #2173, 200 times dilution with 1% BSA PBS) for 2 h followed by secondary antibody (anti-rabbit IgG biotinylated antibody (Cell Signaling Technology, #14708, 1000 times dilution with PBS) or goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, sc-2004, 1000 times dilution with PBS)) for 45 min. After washing by TTBS three times, labeling reactions (4 μM labeling reagent, 200 μM H_2O_2, 10 mM Tris buffer) were performed on sections for 10 min, and sections were washed by TTBS three times, treated with streptavidin-Texas Red (Vector Laboratories) for 30 min. After washing by TTBS three times and PBS once, samples were treated with Hoechst 33342 solution (Dojindo) for 15 min, washed by TTBS twice, and enclosed with VECTASHIELD mounting medium (VECTOR Laboratories). Fluorescence images were observed by fluorescence microscope.

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.xxxx.xx.xxx.

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16. These oxidations are observed only when the phenylenediamine derivatives were used as labeling agents, and they are not observed with other labeling agents such as MAUra. The $+O_n$ peaks are also observed when $Ru(bpy)_3Cl_2$ was used, which suggests that it is an oxidation in the ruthenium complex moiety (See Supporting Information Figure S9).

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24. Compounds **1**, **2**, **3**, **14** and **16** showed no cytotoxicity to living cells. In MTT assay using A549 cells (48 h incubation), no cytotoxicity was observed even when a 1 mM solution was treated for 48 h.

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