Quantitative Interrelation between Atractylenolide I, II, and III in *Atractylodes japonica* Koidzumi Rhizomes, and Evaluation of Their Oxidative Transformation Using a Biomimetic Kinetic Model

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ABSTRACT: Analytical methods based on ultraperformance liquid chromatography/ion-trap mass spectrometry (UPLC/ion-trap MS) were developed for quantification of atractylenolide I, II, and III in the methanol extract of *Atractylodes japonica* rhizomes with a C18 column in an acidified water/acetonitrile gradient eluent in an LC system, and ion-trap MS coupled with electrospray ionization was employed under positive-ion mode. The three atractylenolides were quantified in all *A. japonica* samples, and the content of atractylenolide I, II, and III showed a significant correlation to each other. Such high correlation was explained by the mechanistic insights into the biosynthetic pathway of atractylenoide III and I from atractylenoide II by using the biomimetic cytochrome P450 model, [Fe(tmp)](CF3SO3)-tetramesitylporphyrin). Atractylenolides could be transformed by oxidation via the oxidative enzyme in the *A. japonica* plant. The present study first reports the first oxidative transformation of atractylenolides using the heme iron model complex.

1. INTRODUCTION

The plant *Atractylodes japonica* Koidzumi is distributed in Korea, northeastern China, and Japan, and its dried rhizome has been used to improve gastrointestinal mobility by the induction of distal colon contraction and increasing colonic transit time.1,2 Atractylenolide I, II, and III, which are sesquiterpene-type bioactive compounds in *A. japonica* rhizomes, also exhibit gastrointestinal activities both pharmacologically and clinically: atractylenolide I has been shown to offer improved treatment of gastric cancer and gastric cancer cachexia, and the stimulation of intestinal epithelial cell migration and proliferation, and atractylenolide III has shown gastroprotective activity against acute gastric ulcers.3–6 Hence, it is thought that atractylenolides in *A. japonica* rhizomes play a key role in the treatment of gastrointestinal diseases.

Many analytical methods have been developed for the simultaneous determination or quantification of atractylenolides in *Atractylodes* rhizomes. High-performance liquid chromatography (HPLC)-diode array detection methods have been widely employed for the quantification of atractylenolides II and III in *Atractylodes macrocephala* rhizomes and atractylenolides I and III in *A. japonica* rhizomes.7–9 Gas chromatography–mass spectrometry (MS) has also been used for the determination of atractylenolides I and III in *A. macrocephala* rhizomes.10,11 Being regarded as sensitive and efficient techniques, the LC/triple quadrupole MS method was adopted to identify and quantify atractylenolides in the rhizomes of *A. japonica*, *A. macrocephala*, *Atractylodes lancea*, and *Atractylodes chinensis*.12 Recently, the identification and quantification of atractylenolide I and III in the *A. macrocephala* rhizome by using LC/ion-trap MS has been reported.13 Ion-trap MS can be used to isolate and accumulate ions through the application of characteristic electrodes and oscillating electric fields, and the technique is advantageous for inducing ion fragmentation and accumulation with higher resolution by collision-induced decomposition.14–16 By using these advantages, ion-trap MS has been applied for the identification and quantification of chemical components in herbal medicines.17–19 Nevertheless, the quantitative analysis of atractylenolides I, II, and III in *A. japonica* rhizomes using LC/MS, especially ion-trap MS, has not been reported.

Received: August 13, 2018
Accepted: October 23, 2018
Published: November 5, 2018
cation radical complex, \([\text{tmp}^+\text{Fe}^{IV}(\text{O}))^+]\). The reaction of atractylenolides II by the iron(IV)-oxo porphyrin performed kinetic and mechanistic studies on the hydroxylation of atractylenolides II, with the focus on the iron(IV)-oxo porphyrin complexes. Pearson's correlation coefficient was calculated to evaluate the correlation between the amounts of the three marker compounds and the internal standard (IS) in A. japonica rhizome samples. A Thermo Hypersil GOLD C18 column (2.1 mm × 50 mm, 1.9 μm) was chosen because it provided better ionization of the marker compounds and thus high signal intensity, as previously reported. The flow rate of the gradient elution was 250 μL/min as an optimal eluent for ionization of the marker compounds and the IS in electrospray ionization (ESI) mode. Tandem mass scans were conducted simultaneously, and the peak of each marker compound was baseline separated (Figure S1, Supporting Information).

### 2.2. Optimization of Mass Spectrometric Conditions

To determine the optimal ESI conditions for the ion-trap MS measurements, each marker compound was directly injected into MS in both negative and positive ionization modes. Higher signal intensity and good sensitivity were observed in positive ionization mode than in negative ionization mode, as shown previously. Thus, positive-ion mode was selected for analysis of the three marker compounds in the A. japonica samples. Different conditions such as sheath gas (30, 40, and 50 arb units), auxiliary gas (10, 20, and 30 arb units), capillary temperature (270–300 °C), and capillary voltage (20, 30, and 40 V) were compared. The highest intensity of protonated molecular ions and fragmented ions was obtained under the following conditions: sheath gas, 50 arb units; auxiliary gas, 20 arb units; capillary temperature, 280 °C; and capillary voltage, 30 V.

The protonated molecular ion \([M + H]^+\) of atractylenolide III \((m/z 249.00)\) lost an \(\text{H}_2\text{O}\) molecule and was converted into a fragment ion \((m/z 231.03)\), which was observed with the highest intensity. The protonated molecular ion of atractylenolide II \((m/z 233.00)\) also lost an \(\text{H}_2\text{O}\) molecule and was converted into a fragment ion with \(m/z 215.07\), which was observed with the highest intensity. Unlike the two compounds discussed above, the protonated molecular ion of atractylenolide I \((m/z 231.00)\) was mainly converted into a fragment ion \((m/z 185.09)\). The protonated molecular ion of bergapten (IS, \(m/z 217.00\)) lost a \(\text{CH}_3\) molecule and was converted into a fragment ion.

### 2. RESULTS AND DISCUSSION

#### 2.1. Optimization of Chromatographic Conditions

Optimal conditions for chromatographic analysis were established by comparing the column, mobile phase, mobile phase modifier, and flow rate to separate and detect the three marker compounds and the internal standard (IS) in A. japonica samples. A Thermo Hypersil GOLD C18 column (2.1 mm × 100 mm, 1.9 μm) was chosen because it afforded improvement of the peak shape and better resolution within 15 min, compared with a Thermo Hypersil GOLD C18 column (2.1 mm × 50 mm, 1.9 μm). The mobile phase consisted of water and acetonitrile, with 0.1% formic acid being added for better ionization of the marker compounds and thus high signal intensity, as previously reported. The flow rate of the gradient elution was 250 μL/min as an optimal eluent for ionization of the marker compounds and the IS in electrospray ionization (ESI) mode. Tandem mass scans were conducted simultaneously, and the peak of each marker compound was baseline separated (Figure S1, Supporting Information).

### Table 2. Recovery of Three Marker Compounds in the Methanol Extract of A. japonica Rhizomes (n = 3)

| compound          | initial concentration (μg/mL) | spiked concentration (μg/mL) | detected concentration (μg/mL) | recovery (%) | RSD (%) |
|-------------------|------------------------------|------------------------------|--------------------------------|--------------|---------|
| atractylenolide III | 8.22                         | 1.88                         | 9.90                           | 89.56        | 9.12    |
|                   | 3.75                         | 1.25                         | 12.00                          | 100.64       | 13.95   |
|                   | 7.50                         | 1.57                         | 15.87                          | 101.93       | 8.91    |
| atractylenolide II | 8.20                         | 1.25                         | 9.36                           | 93.15        | 12.29   |
|                   | 2.50                         | 1.06                         | 10.61                          | 96.45        | 11.19   |
|                   | 5.00                         | 1.13                         | 13.11                          | 98.34        | 6.66    |
| atractylenolide I | 11.20                        | 1.88                         | 12.76                          | 83.50        | 12.74   |
|                   | 3.75                         | 1.49                         | 14.49                          | 87.75        | 13.47   |
|                   | 7.50                         | 1.83                         | 18.36                          | 95.51        | 7.25    |

"RSD (%) = (SD/mean) × 100.

### Table 1. Intra- and Interday Precisions of Three Marker Compounds in the Methanol Extract of A. japonica Rhizomes

| compound          | initial concentration (μg/mL) | in day (n = 3) | detected concentration (μg/mL) | RSD (%) | accuracy (%) | interday (n = 3) | detected concentration (μg/mL) | RSD (%) | accuracy (%) |
|-------------------|------------------------------|---------------|--------------------------------|---------|--------------|-----------------|--------------------------------|---------|--------------|
| atractylenolide III | 0.94                         | 0.86          | 12.32                          | 91.74   |              | 0.86            | 12.03                          | 91.42   |              |
|                   | 3.75                         | 4.27          | 2.10                           | 113.94  |              | 4.34            | 4.64                           | 115.70  |              |
|                   | 15.00                        | 15.87         | 4.05                           | 105.82  |              | 16.26           | 5.73                           | 108.42  |              |
| atractylenolide II | 0.63                         | 0.59          | 1.97                           | 93.66   |              | 0.52            | 10.62                          | 82.85   |              |
|                   | 2.50                         | 2.72          | 2.52                           | 108.97  |              | 2.73            | 4.77                           | 109.10  |              |
|                   | 10.00                        | 10.47         | 4.89                           | 104.66  |              | 11.05           | 7.82                           | 110.52  |              |
| atractylenolide I | 0.94                         | 0.86          | 5.89                           | 91.29   |              | 0.81            | 9.34                           | 86.93   |              |
|                   | 3.75                         | 4.07          | 2.50                           | 108.46  |              | 4.05            | 3.19                           | 107.89  |              |
|                   | 15.00                        | 15.56         | 5.03                           | 103.73  |              | 16.04           | 6.56                           | 106.95  |              |

"RSD (%) = (SD/mean) × 100.

Meanwhile, in cytochromes P450 (CYP450) is present in plants, high-valent iron(IV)-oxo porphyrin π-cation radicals, referred to as compound I (Cpd I), have been proposed as reactive intermediates mainly involved in the biosynthetic pathway of sesquiterpenes. For example, the hydroxylation of alkanes by Cpd I is initiated by a hydrogen atom abstraction (HAT) step, followed by an oxygen rebound step. However, kinetic and mechanistic insights into the hydroxylation of atractylenolides by Cpd I have not yet been evidenced.

In the present study, three marker compounds of the A. japonica rhizome, atractylenolides I, II, and III, were quantified in the methanol extract of A. japonica samples obtained from different locations in Korea and China using LC/ion-trap MS. Moreover, the correlation between the amounts of the three marker compounds and the internal standard (IS) in A. japonica samples was evaluated by calculating Pearson's correlation coefficient. Moreover, we performed kinetic and mechanistic studies on the hydroxylation of atractylenolides II by the iron(IV)-oxo porphyrin π-cation radical complex, \([\text{tmp}^+\text{Fe}^{IV}(\text{O}))^+]\).
The fragmentation of protonated fragment ion (m/z 201.98), which was consistent with previous results (Figure S2, Supporting Information). The fragmented product ions of each compound, m/z 231.03, 215.07, and 185.09 for atractylenolides III, II, and I, respectively, were selected for identification and quantification of the marker compounds in the methanol extracts of A. japonica samples.

2.3. Method Validation. The correlation coefficients ($r^2$) of each linear equation were in the range of 0.9976–0.9999 within the linear ranges: 0.47–30.00 μg/mL for atractylenolide III, 0.31–20.00 μg/mL for atractylenolide II, and 0.47–30.00 μg/mL for atractylenolide I. LODs and LOQs were 23–43 and 78–146 ng/mL, respectively (Table S1, Supporting Information). The precision estimates of the LC/MS method determined for three concentrations of marker compounds were 1.97–12.32% of the relative standard deviation (RSD) value within one day and 3.19–12.03% of the RSD value during three days (Table 1). Recovery of added compounds ranged from 83.50 to 101.93%, with the RSD value less than 14.00% (Table 2). The repeatability, represented as RSD values, was 4.63–6.04% for the absolute peak area and 0.06–0.62% for the retention time (Table S2, Supporting Information).

These results indicate that the LC/MS method developed here allows linear, sensitive detection of each compound and that precise, accurate, and reliable measurements can be obtained for the quantification of the three marker compounds in the methanol extracts of A. japonica rhizomes.

2.4. Quantification of the Three Marker Compounds in A. japonica Samples. The fragmentation of protonated ion molecules to the most intense fragment ions of m/z 249.00 $\rightarrow$ 231.03 for atractylenolide III, m/z 233.00 $\rightarrow$ 215.07 for atractylenolide II, and m/z 231.00 $\rightarrow$ 185.09 for atractylenolide I were employed for quantification in A. japonica samples. The average content of the three marker compounds varied across the A. japonica samples. The amount of atractylenolide III was highest, followed by atractylenolides I and II in most samples with significance, which is consistent with the previous study, although the no. 25 sample showed no significant difference in contents. In addition, the differences in the amounts (highest amount/lowest amount) of each compound were from 11- to 26-fold within A. japonica samples (Table S3, Supporting Information).

A diverse range of environmental circumstances, including location, climate, wild/cultivated, seasons, and soil composition, is speculated to determine the production or accumulation of sesquiterpenes in Atractylodes rhizomes because the occurrence of secondary metabolites is mainly affected by such extrinsic factors. Moreover, the defense system of the Atractylodes rhizome against pathogens can also influence (mainly increase) sesquiterpene accumulation through the induction of jasmonic acid, which is a plant-signaling molecule in defense response. It is assumed that those environmental and defensive factors result in various contents of atractylenolides in A. japonica rhizomes in different locations.

2.5. Correlation between the Average Content of the Three Marker Compounds in A. japonica Samples. Correlations between the marker compounds based on the average content measured in A. japonica samples were evaluated by using Pearson’s correlation coefficient ($r$), which is a value that ranges from −1 to +1. Pearson’s correlation coefficient is generally used to evaluate the degree of linear interrelation between two variables and measures an influence of one variable on the other variable.
A. japonica plant A. lancea participates in the formation of diverse sesquiterpenoids in CYP450, catalases, and peroxidases. As CYP450 activating catalytic cycles of heme iron enzymes, such as coe have frequently been invoked as key intermediates in the O2-π π
cation radical species through oxygen exchange with 18O- labeled water. The conversion of atractylenolide III to atractylenolide I via dehydration was also detected when the reaction of atractylenolides. Upon addition of atractylenolide II to the solution of I, the absorption band at 665 nm due to [FeIII(tmp)][CF3SO3] resulted in the formation of I at -40 °C (Figure 2a). We then explored the reactivities of I in the C–H bond activation reaction of atractylenolides. Upon addition of atractylenolide II to the solution of I, the absorption band at 665 nm due to I disappeared with a concomitant increase of the absorption band at 505 nm because of [FeIII(tmp)][CF3SO3] at -40 °C. The decay rate was increased linearly with the increase of atractylenolide II concentrations; second-order rate constants (k2) of 1.5 × 10-1 M-1 s-1 was determined (Figure 2b).

Very interestingly, the product analysis of the reaction solution of the oxidation of atractylenolide II by I revealed that atractylenolide III was produced as a sole product with a quantitative yield (>95% based on the amount of I used). When the atractylenolide II oxidation was performed with 1 in the presence of H218O, atractylenolide III contained 90% of 18O under the identical reaction conditions (Figure 3). This result clearly indicates that the source of oxygen in the hydroxylated product was the iron(IV)-oxo porphyrin π–cation radical species through oxygen exchange with 18O-labeled water. The conversion of atractylenolide III to atractylenolide I via dehydration was also detected when the resulting solution was kept under an air atmosphere for several hours.

We also characterized the iron product formed in the atractylenolide II oxidation by 1. The negative mode ESI-MS spectrum of the reaction solution exhibited a prominent peak...
Taken together, the hydroxylation of atractylenolide II by formation of [FeIII(tmp)]+ and atractylenolide III. Dehydration followed by an oxygen rebound process, resulting in the oxidation of atractylenolide II by I. When the resulting reaction solution was kept for several hours under an air atmosphere, a decrease of the UV–vis absorbance at 505 nm was observed (Figure 2A, blue line) upon addition of 40 equiv of atractylenolide II at −40 °C resulting in the formation of [FeIII(tmp)](CF3SO3) (red line). Inset shows the time course of the decay of atractylenolide III to atractylenolide I was also observed when the resulting reaction solution was kept for several hours under an air atmosphere. The [FeIII(tmp)]+ (calculated m/z of 836.8) was assigned as [Fe(tmp)]+ (calculated m/z of 836.9). Inset shows the isotopic distribution patterns of [Fe(tmp)]+.

3. CONCLUSIONS

An efficient, accurate, and precise analytical LC/ion-trap MS method was developed and applied for the quantification of atractylenolides I, II, and III in A. japonica rhizomes with a validation in terms of linearity, recovery, precision, and repeatability. The positive ionization mode was employed for the detection of molecular ion fragmentation at m/z 249.00 → 231.03 for atractylenolide III, m/z 233.00 → 215.07 for atractylenolide II, and m/z 231.00 → 185.09 for atractylenolide I. The average contents of the three atractylenolides were determined in A. japonica samples obtained from different locations in Korea and China. The results reveal a variation in the amounts of analytes among the samples, and high correlations were observed between the content of the atractylenolides in the samples. A significant correlation between atractylenolides was evidenced by the CYP450-mimetic oxidation model. Finally, we also provided (i) kinetic value of oxidative transformation of atractylenolide II by high-valent iron(IV)-oxo porphyrin π–cation radical species and (ii) experimental evidence that the hydroxylation of atractylenolide II occurs via hydrogen atom abstraction, followed by an oxygen rebound mechanism. These results would be helpful to understand the existence and role of oxidative enzymes, especially cytochrome P450, during the biosynthesis in the A. japonica plant.

4. EXPERIMENTAL SECTION

4.1. Chemicals and Reagents. Commercially available reagents were of the best available purity and were used without further purification unless otherwise noted. LC/MS-grade acetonitrile, water, and formic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade methanol was obtained from J.T. Baker Inc. (Phillipsburg, NJ, USA). Acetonitrile (CH3CN) and dichloromethane (CH2Cl2) were dried according to the literature procedures and distilled under Ar prior to use. 99–m-CPBA was purified by washing with phosphate buffer (pH 7.4) followed by water and then dried under reduced pressure. [FeIII(tmp)]Cl was purchased from Frontier Scientific Inc. (Logan, UT, USA). [FeIII(tmp)](CF3SO3) was prepared by stirring equimolar amounts of [FeIII(tmp)]Cl and AgCF3SO3 followed by filtration through a 0.45 mm filter; the resulting solution was used immediately. Atractylenolides I and III were purchased from Biomart (Guangzhou, Guangdong, China). Atractylenolide II was purchased from Chengdu Biopurify Phytochemicals (Chengdu, Sichuan, China). Bergapten (IS) was purchased from ChemFace (Wuhan, Hubei, China). All marker compounds had purities of ≥98%.

Forty-one samples of A. japonica rhizomes were either purchased from herbal companies or harvested from the wild...
fields in Korea and China. The samples were authenticated by the authors (Table S4, Supporting Information). Voucher specimens (2016-PNUKMAJ-01-41) have been deposited at the School of Korean Medicine, Pusan National University.

4.2. Sample Preparation. The *A. japonica* rhizomes were pulverized and passed through a 500 μm testing sieve (Chunggyesanggong-sa; Gunpo, Gyeonggi, Korea) to produce a homogenized powder. Accurately weighed powder (0.5 g) was extracted into 10 mL of methanol (w/v) for 30 min using an ultrasonic extractor (Power Sonic S20; Hwashin Tech, Daegu, Korea). The extracted solution was centrifuged at 10,000 rpm for 5 min. The supernatant was transferred to a 1.5 mL polypropylene tube and then evaporated using a nitrogen-blowing concentrator (MGS2200; EYELA, Miyagi, Japan). The concentrated extract was dissolved in HPLC-grade methanol to a concentration of 1000 μg/mL and filtered through a 0.2 μm syringe filter (BioFact, Daejeon, Korea) prior to LC/MS injection.

4.3. Analytical Apparatus. An Accela LC system (Thermo Fisher Scientific; CA, USA) equipped with a refrigerated autosampler, degasser, and quaternary solvent pump was employed for the quantitative analysis. The three marker compounds were separated on a Hypersil GOLD C18 column (2.1 mm × 100 mm, 1.9 μm; Thermo Fisher Scientific, CA, USA) at 35 °C. The flow rate was 250 μL/min, and the injection volume was 5 μL. The mobile phase consisted of water (containing 0.1% formic acid; A) and acetonitrile (B), and an isocratic elution was performed with an A/B ratio of 45:55 (v/v) until the end of the analysis (to 15 min).

An LCQ Fleet ion-trap mass spectrometer (Thermo Fisher Scientific; CA, USA) was used to analyze the compounds in the LC eluent, which was introduced into the ESI source in positive-ion mode. Instrumental conditions were as follows: sheath gas (nitrogen), 50 arb units; auxiliary gas (nitrogen), 20 arb units; spray voltage, 5.0 kV, capillary temperature, 280 °C; and capillary voltage, 30.0 V. Tandem mass scans (MS2) from the protonated molecular ion [M + H]+ were employed for quantification of the marker compounds and the IS. The collision energy was set at 35% for collision-induced dissociation in the MS2 experiment. Data were processed using Xcalibur software (v. 2.1.0; Thermo Fisher Scientific, CA, USA).

UV–vis spectra were recorded on a Hewlett Packard Agilent 8454 UV–visible spectrophotometer equipped with a Unisoku cryostat system (USP-203; Unisoku, Japan). Product analysis was performed with Agilent Technologies 1220 Infinity II HPLC.

4.4. Method Validation. The three marker compounds were accurately weighed and dissolved in methanol at 1000 μg/mL to make stock solutions, which were diluted to produce seven working solutions containing the IS. The calibration curves were constructed by plotting the peak area ratio of the three marker compounds to the IS (y-axis) versus the concentration of the three marker compounds in the working solution (x-axis). The linearity of each calibration curve was evaluated by the correlation coefficients (r²). LOD and LOQ were determined as signal-to-noise (S/N) ratios of 3 and 10, respectively. Precision was determined by analyzing low, middle, and high concentrations of working solutions of the marker compounds three times within a day (intraday precision) and during three consecutive days (interday precision). Values of precision, as indicated as RSD, as follows: RSD (%) = [(standard deviation/mean) × 100]. The accuracy of the LC/MS method used was evaluated by recovery testing. Three known amounts of marker compounds (low, middle, and high) were added to the methanol extract of *A. japonica* samples, and the recovery was calculated as follows: recovery (%) = [(detected concentration – initial concentration)/spiked concentration] × 100.

4.4.1. Repeatability. The repeatability was indicated as RSD values of retention times and RSD values of the absolute areas of the marker compounds, based on six replicate analyses of a sample.

4.5. Statistical Analysis. Multiple comparisons between the contents of atractylenolide I–III in each *A. japonica* sample were performed by using Tukey’s test. Differences were considered significant at p < 0.05, p < 0.01, or p < 0.001. Pearson’s correlation coefficient of the average content of atractylenolide I–III in the *A. japonica* samples was calculated with significance of p-values < 0.001. Multiple comparisons and Pearson’s correlation coefficient were calculated by using open-source software R (v. 3.4.3; The R Foundation for Statistical Computing).

4.6. Kinetics Studies. Reactions were followed by monitoring UV–vis spectral changes of reaction solutions at −40 °C. All reactions were run, at least, in triplicates, and the data reported represent the average of these reactions. Iron(IV)-oxo porphyrin π-cation radical species, [(tmp+)·Fe(IV)(O)]+(1), was prepared by literature methods.40 The formation of the iron-oxo intermediates was confirmed by UV–vis spectroscopy. Subsequently, appropriate amounts of atractylenolide II were added to the reaction solutions. After the completion of reactions, pseudo-first-order fitting of the kinetic data allowed us to determine kobs values. The resulting solution was directly injected to HPLC in order to identify and quantify the reaction products. Products were determined by comparing retention times and mass patterns to those of known authentic samples. Atractylenolide III (>95%) was formed as a sole product. Product yields were determined by comparison against standard curves prepared with authentic samples.

ASSOCIATED CONTENT

3 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02005.

Tables S1–S4 showing calibration curves, repeatability, and the average contents of atractylenolide I, II, and III and the sample list of *A. japonica* rhizomes and figures S1 and S2 showing extracted ion chromatograms of atractylenolide I, II, and III and their mass spectra (PDF)

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Notes

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ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government through MSIP (NRF-2015R1C1A1A01053466 to J.-H.K.), (NRF-2017R1C1B2002037 to S.H.), and Sookmyung Women’s University Grants (1-1703-2038 to S.H.).

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