Repellency of zerumbone identified in *Cyperus rotundus* rhizome and other constituents to *Blattella germanica*

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The compounds 1,8-cineole and zerumbone (ZER) from the *Cyperus rotundus* rhizome along with another 11 previously identified rhizome essential oil constituents and α-humulene, which lacks the only carbonyl group present in ZER, as well as binary mixtures of ZER and seven active compounds were tested for repellency to male *B. germanica*. The results were compared to *N*,*N*-diethyl-3-methylbenzamide (deet). In filter-paper choice tests, ZER was the most repellent compound, and α-humulene was ineffective, which indicates that the α,β-unsaturated carbonyl group of ZER is a prerequisite component for repellency. At 81.5 μg cm⁻², enhanced repellency was produced by binary mixtures of ZER and 1,8-cineole, (+)-dihydrocarvone or (R)-(+)limonene (70:30, 50:50 and 30:70 ratios by weight). These mixtures were very effective against male *B. germanica* within 24 h and were more repellent than a single compound or deet alone. The optimum ZER content was determined to be more than 50%. In Ebeling choice box tests at 652.4 μg cm⁻², these compounds and deet resulted in complete repellency to intact male *B. germanica*, while they exhibited 35–47% repellency to antennectomized male one. Mixtures formulated from the active constituents of the *C. rotundus* rhizome could be useful as potential repellents for controlling *B. germanica*.

The German cockroach, *Blattella germanica* L. (Blattodea: Blattellidae), is one of the major sources of potent allergens in sensitive populations¹–³. Approximately 43% of the United States (US) population (6–59 years old) is allergic to at least one common indoor allergen, and 26% are sensitive to *B. germanica⁴*. Cockroach allergens are associated with feces, saliva, secretions and fragments of their body parts⁵. The prevalence and severity of allergic diseases, such as asthma and rhinitis, caused by cockroaches are increasing, especially among children⁶,⁷, and the diseases are some of the most serious global public health problems⁶–⁸. In addition, cockroach exuviae can support large populations of the European house dust mite, which is an important producer of various allergens that lead to exacerbated cases of bronchial asthma⁹.

Cockroach repellents can be used as a preventive tool in the transport and storage of merchandise, protecting sensitive areas such as kitchens, children’s nurseries and hospitals, and reaching inaccessible cockroach hiding places, such as crevices, electrical ducts, plumbing ducts and cabinet voids, as well as for protecting sensitive electronic equipment, communications equipment and food industry facilities⁴. The most widely used insect repellent products are currently based on *N*,*N*-diethyl-3-methylbenzamide (deet)¹⁰, which continues to be an effective compound. However, deet has many problems, such as having an unpleasant odor and causing damage to certain plastics and synthetic rubber as well as causing medical issues such as central nervous system depression, urticaria and potential encephalopathic toxicity¹¹. In addition, the number of approved insecticides and repellents may be reduced in the near future in the US by the US Environmental Protection Agency as re-registration occurs under the 1996 Food Quality and Protection Act¹². The removal of conventional insecticide or repellent products from markets due to the increase in insecticide resistance or welfare concerns will have a serious impact on the proliferation of *B. germanica¹³,¹⁴*. Therefore, there is a pressing need for the development of eco-friendly alternatives for the control of *B. germanica*, particularly alternatives with repellency because many insecticides

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are difficult to apply to inaccessible places, do not reach deep, insecticide-free harborages and cannot be applied to sensitive environments9.

Biorepellents and botanical insecticides derived from plants may provide potential alternatives for household and medically important pest insects because plants are a potential source of bioactive secondary metabolites that are perceived by the general public as relatively safe and pose fewer risks to the environment with minimal impacts on human health15–18. Many efforts have been focused on plants as potential sources of commercial repellents, in part, because certain plant preparations meet the criteria of minimum-risk repellents 19. Previous studies have shown that the methanol extract and essential oil from the rhizome of coco-grass, *Cyperus rotundus* L. (Poales: Cyperaceae), possessed good repellency against male *B. germanica*20. Limited information is available concerning the potential uses of the *C. rotundus* rhizome and its constituents for the control of *B. germanica*, despite the insecticidal properties of the plant20.

In this study, our aim was to assess whether the two monoterpenoids, 1,8-cineole and zerumbone (ZER), which were extracted from *C. rotundus* rhizomes, and another 11 previously identified constituents without insecticidal activity from the rhizome essential oil20 had repellent activity against male *B. germanica* compared to deet, a positive control, in a filter-paper choice bioassay. Deet is registered as an insect repellent in South Korea21.

The repellency of binary mixtures of ZER and seven active compounds was also evaluated. Finally, the repellency of α-humulene was examined to discern whether the α,β-unsaturated carbonyl group of ZER is essential for repellent activity. The relationship between repellency and boiling points (BPs) of the test compounds is also discussed.

### Results

#### Bioassay-guided fractionation and isolation.

The fractions obtained from solvent partitioning of the methanol extract of the *C. rotundus* rhizome were tested against male *Blattella germanica* using a filter-paper choice assay at 244.6 μg cm^-2. Means within a column followed by the same letter are not significantly different (*P = 0.05, Bonferroni method).

| Material                        | Repellency (%) (± SE) |
|---------------------------------|-----------------------|
| Methanol extract                | 100±a                 |
| Hexane-soluble fraction         | 100±a                 |
| Chloroform-soluble fraction     | 6±1.6b                |
| Ethyl acetate-soluble fraction  | 8±2.2b                |
| Water-soluble fraction          | 8±2.4b                |

Table 1. Repellency of fractions obtained from the hydrolyzable solvent of the methanol extract of *Cyperus rotundus* rhizome against male *Blattella germanica* using a filter-paper choice assay at 244.6 μg cm^-2. Means within a column followed by the same letter are not significantly different (*P = 0.05, Bonferroni method).

Figure 1. Structures of 1,8-cineole, zerumbone and α-humulene. 1,8-Cineole and zerumbone were identified in the *Cyperus rotundus* rhizome in this study. The chemical formula of 1,8-cineole (1) is C10H18O with a molar mass of 154.249 g/mol. The chemical formula of zerumbone (2) is C15H22O with a molar mass of 218.335 g/mol. α-Humulene (3) is a zerumbone analog that lacks the α,β-unsaturated carbonyl group.

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### Results

#### Bioassay-guided fractionation and isolation.

The fractions obtained from solvent partitioning of the methanol extract of the *C. rotundus* rhizome were tested against male *B. germanica* using a filter-paper choice assay (Table 1). Significant differences in repellency were observed among the fractions and were used to identify the peak activity fractions for the next step in purification. At a concentration of 244.6 μg cm^-2, the hexane-soluble fraction was the most potent repellent, while no repellency was obtained using the chloroform-, ethyl acetate- or water-soluble fractions.

Filter-paper choice bioassay-guided fractionation of the *C. rotundus* rhizome led to two active compounds that were identified through spectroscopic analyses, including electron ionized mass spectrometry (EI-MS) and nuclear magnetic resonance (NMR) spectroscopy. The two repellent compounds were 1,8-cineole (1) and zerumbone (2) (Fig. 1). 1,8-Cineole (1) was identified based on the following evidence: colorless oil; EI-MS (70 eV), m/z (% relative intensity): 154 [M]+ (98), 139 (68), 126 (18), 108 (93), 93 (49), 81 (100), 71 (80), 69 (61), 55 (53),
In this study, the repellency of 1,8-cineole and ZER from the C. rotundus rhizome along with 11 previously identified constituents and binary mixtures of ZER and seven active compounds against male B. germanica was assessed. The repellency of these materials was compared to deet, which is a currently available synthetic repellent, to determine whether they would be suitable for future commercial cockroach repellents. Historically, C.
of the tested compounds, ZER was the most potent repellent. Potent repellency was also produced using a filter-paper choice box assay at 81.5 μg cm⁻². Table 3. Residual repellency of eight selected organic pure compounds and the commercial repellent deet against male Blattella germanica using an Ebeling choice box assay at 652.4 μg cm⁻². Means within a row followed by the same lowercase letter in the same column or the same uppercase letter in the same row are not significantly different (P = 0.05, Bonferroni method).

Table 4. Residual repellency of binary mixtures of zerumbone and seven active compounds against male Blattella germanica using a filter-paper choice box assay at 81.5 μg cm⁻² for 24 h. Means within a row followed by the same letter are not significantly different (P = 0.05, Bonferroni method).

Table 5. Repellency of five pure organic compounds and commercial repellent deet to antennectomized and intact male Blattella germanica using an Ebeling choice box assay at 652.4 μg cm⁻².

rotundus has been widely used as an analgesic, a sedative, an antiplasmodic agent, an antimalarial and a treatment for stomach disorders. Many plant extracts and essential oils produce repellency to different insect species. Repellent constituents derived from plants against cockroaches include flavonoids, phenylpropanoids and terpenoids. For example, the monoterpenoids, E.Z- and Z,E-nepetalactone as well as pulegol, isopulegol and pulegone work against B. germanica, and the phenylpropanoids, safrole and isosafrole, work against Periplaneta americana (L.) (Blattodea: Blattidae) and have been reported as repellent compounds. In the current study, the repellent constituents of the C. rotundus rhizome were determined to be the sesquiterpenoid ZER and the monoterpenoid 1,8-cineole.
Volatile constituents derived from most plant extracts and essential oils consist of alkanes, alcohols, aldehydes and terpenoids (particularly monoterpenoids) and some are insect repellents. Because of their high volatility, the essential oils and their constituents are usually effective against arthropods only for a relatively short period, which is typically less than 2 h. However, available information on B. germanica repellency is limited because most previous studies that investigated repellency used a bioassay with a short time period, which was mainly 5 to 10 min. The compounds 1,8-cineole and α-pinene have been reported to have 92 and 82% repellency, respectively, against B. germanica nympha after a 2 h post-treatment at a concentration of 5 ppm. However, Appel et al. reported that mint oil extract at the surface concentration of 2 mL of mint oil to 457.5 cm² was extremely repellent (approximately 100%) to adult males of B. germanica and P. americana for 14 days.

In the current study with Ebeling choice box tests, (−)- (E)-pinocarveol and ZER were very effective against male B. germanica for 8–10 h, while (−)-dihydrocarvone, β-caryophyllene and (1S)-(−)-verbenone were very effective for 4 h. Nevertheless, these compounds were less potent repellents than deet. The differences in repellency could be attributed to the differences in the quantitative losses because of the different volatility of the compounds, as described by Brown and Hebert. The ability of a chemical vapor to function as a repellent has been reported to be related to its BP, and BPs between 230 and 260 °C at atmospheric pressure were found to be the most desirable for an effective repellent. The BPs of (E)-carveol and (−)-α-copaene coincide with the optimal range but were less effective than either ZER with higher BPs, compounds with lower BPs or compounds with similar BPs. Thus, other factors, such as structural characteristics (e.g., types of functional groups and carbon skeletons), rather than BP appear to be associated with repellency of these compounds against male B. germanica. For example, β-caryophyllene was more effective than caryophyllene oxide. Interestingly, α-humulene, which lacks only the α,β-unsaturated carbonyl group present in ZER, was virtually ineffective. This finding indicates that the α,β-unsaturated carbonyl group of ZER is a prerequisite component for repellency to B. germanica. Peterson et al. studied the repellency of catnip, Nepeta cataria L. (Lamiaceae: Lamiaceae), as well as its essential oil constituents, against male B. germanica. They reported that the iridoid monoterpoid, E.Z.-nepeta lactone, was significantly more active than the Z,E-isomer. In addition, ZER, (−)-dihydrocarvone, β-caryophyllene, (1S)-(−)-verbenone, 1,8-cineole and deet produced complete repellency against intact male B. germanica, while these compounds exhibited 35–47% repellency against antennectomized male B. germanica. These results indicate that antennae are partly responsible for the perception of the tested compounds. Detailed tests are needed to fully understand their modes of repellent action.

It is well known that repellency against various insect species was more pronounced in binary mixtures of phytochemicals compared to the corresponding single compounds. For example, a significantly enhanced repellency was produced through binary mixtures of E.Z- and Z,E-nepeta lactone that were consistently more repellent than the isomers when they were tested alone against Anopheles gambiae Giles (Diptera: Culicidae), expect for equivalent or near-equivalent mixtures, which had significantly lower repellency. In the current study, repellency against male B. germanica produced by the binary mixtures of ZER and 1,8-cineole, (−)-dihydrocarvone or (R)-(−)-limonene at three mixture ratios were more pronounced compared to repellency produced by the corresponding compound and deet alone. The improved effectiveness of repellency could be attributed to the lower evaporation rate and/or better persistence of ZER in the combined presence of another compound, as described by Khan et al. and Tuetun et al. The optimum ZER content was determined to be more than 50% according to our laboratory results. This original finding indicates that binary mixtures could be promising repellent products that are novel and effective against B. germanica. However, these terpenoids are volatile, and the volatility problem may be alleviated by special formulations, such as microencapsulation, that can reduce volatile loss, as described by Peterson et al.

In conclusion, C. rotundus rhizome-derived products containing active compounds, especially zerumbone and 1,8-cineole, could be useful as repellents in the control of B. germanica populations in sensitive environments in which conventional insecticides would be inappropriate, as long as special formulations (e.g., microencapsulation) that facilitate the slow release of active compounds can be selected or developed. Further research is needed for the practical applications of plant-derived preparations as novel cockroach repellent products to establish their safety profiles in humans. In addition, their effects on non-target organisms and the environment need to be established. Finally, detailed tests are needed to understand how to improve repellency potency and stability for eventual commercial development.

Methods

Institutional analysis. The 1H and 13C NMR spectra were recorded in CD3OD on an Avance 400 WB spectrometer (Bruker, Rheinstetten, Germany) at 400 and 100 MHz, respectively, using tetramethylsilane as an internal standard. The chemical shifts are given in δ (ppm). The DEPT spectra were acquired using Bruker software. The mass spectra were obtained on a GCMS-QP 2010 Ultra gas chromatograph–mass spectrometer (Shimadzu, Columbia, MD, USA). Silica gel 60 (0.063–0.2 mm) (Merck, Darmstadt, Germany) was used for column chromatography. Merck pre-coated silica gel plates (Kieselgel 60 F254) were used for analytical thin layer chromatography (TLC). A Spectra System P 2000 high-performance liquid chromatograph (HPLC) (Thermo Scientific, Waltham, MA, USA) was used to isolate the active compounds.

Chemicals. Two constituents, 1,8-cineole and ZER, were identified in this study, and another 11 previously identified constituents from the C. rotundus rhizome essential oil are listed in Table 6 along with their BPs and purities. α-Humulene, which is a zerumbone analog lacking the α,β-unsaturated carbonyl group, was also used in this study for structure-activity relationship (Fig. 1). All compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the relationship between toxicity and BPs of the tested compounds, the BP values of these compounds were obtained from ACD/ChemSketch (ACD/LAB 12.0 for Microsoft Window, Advanced Chemistry
Development, Inc., Montreal, Canada) (Table 1). Deet (97.0% purity) was purchased from Sigma-Aldrich. All of the other chemicals used in this study were reagent-grade quality and are available commercially.

**Cockroaches.** The stock cultures of *B. germanica* (susceptible KSS strain)\(^{30}\) were maintained in the laboratory without exposure to any known insecticide. Cockroaches were reared in glass jars (43-cm diameter \(\times\) 30 cm) containing Purina calf food pellets (Pyongtaek, Gyeonggi, South Korea), distilled water and a cardboard shelter at 27 ± 2 °C and 60 ± 5% relative humidity (RH) under a 12:12 h light-dark cycle. Because male *B. germanica* are more sensitive than females to olfactory stimuli\(^{31,46}\), adult males were used for the repellency bioassays.

**Plant material.** The rhizome of *C. rotundus* was purchased from the Boeun medicinal herb shop (Seoul Yangnyeongsi, Seoul, South Korea). A voucher specimen (CR–01) was deposited in the Research Institute of Agriculture and Life Sciences at Seoul National University.

**Bioassay-guided fractionation and isolation.** Air-dried rhizomes (600 g) of *C. rotundus* were pulverized, extracted with methanol (3 \(\times\) 3 L) at room temperature for 2 days and filtered. The combined filtrate was concentrated by rotary evaporation at 40 °C to yield approximately 152 g of a dark greenish tar. The extract (100 g) was sequentially partitioned into hexane- (16.4 g), chloroform- (45.3 g), ethyl acetate- (10.7 g) and water-soluble portions at 40 °C, and the water-soluble portion was concentrated at 50 °C. To isolate the active constituents, the organic solvent-soluble portions were concentrated under vacuum at 40 °C, and the water-soluble portion was concentrated at 50 °C. To isolate the active constituents, 244.6 \(\mu\)g cm\(^{-2}\) of each *C. rotundus* rhizome-derived fraction were tested in a filter-paper choice bioassay, as described by Petterson et al.\(^{31}\).

The hexane-soluble fraction (15 g) was the most biologically active fraction (Table 1) and was chromatographed on a 5.5 \(\times\) 70 cm silica gel (600 g) column through elution with a gradient of hexane and ethyl acetate [100:0 (2 L), 95:5 (1 L), 90:10 (1 L), 80:20 (1 L) and 70:30 (1 L) by volume] and then elution with methanol (2 L) to provide 40 fractions (each approximately 200 mL) (Fig. 2). The column fractions were monitored by TLC on silica gel plates developed with a hexane and ethyl acetate (7:3 by volume) mobile phase. Fractions with similar *R*\(_t\) values on the TLC plates were pooled. The spots were detected by spraying the plate with 10% sulfuric acid and then heating the samples on a hot plate. Active fractions 1–10 (H1) were obtained. Fraction H1 was re-chromatographed on a 5.5 \(\times\) 70 cm silica gel (600 g) column through elution with a gradient of hexane and ethyl acetate [80:20 (1 L), 70:30 (1 L) and 50:50 (1 L) by volume] and a final elution with methanol (2 L) to provide 25 fractions (each approximately 200 mL). Active fractions 1–5 (H111) were obtained. Fraction H111 was re-chromatographed on a silica gel column by elution with a gradient of hexane and ethyl acetate [90:10 (1 L), 80:20 (1 L) and 70:30 (1 L) by volume] and a final elution with methanol (2 L) to provide 25 fractions (each approximately 200 mL). Active fractions 1–5 (H1111) were obtained.

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**Filter-paper choice assay.** A filter-paper choice test described previously by Petterson et al.\(^{31}\) was used to evaluate the repellency of all compounds against male *B. germanica* (7–10 days old). Each test was conducted between 20:00 to 23:00 h with overhead florescent lighting at 27 ± 2 °C and 60 ± 5% RH in 5-min test periods, as described previously\(^{31}\). A 12.5-cm diameter Whatman no. 2 filter paper (Whatman, Maidstone, UK) was cut in

| Compound          | Boiling point (°C/760 mmHg) | Purity (%) |
|-------------------|----------------------------|------------|
| (E)-carveol       | 231.46                     | ≥95.0      |
| β-caryophyllene   | 268.36                     | ≥98.5      |
| (−)-Caryophyllene oxide | 279.68         | 95.0      |
| 1,8-Cineole       | 174.01                     | 99.0       |
| (−)-α-copaene     | 248.50                     | ≥90.0      |
| (−)-Dihydrocarveol| 221.50                     | 98.0       |
| α-humulene        | 276.35                     | ≥98.0      |
| (R)-(++)-Limonene | 175.44                     | ≥93.0      |
| (S)-(−)-Limonene  | 175.44                     | ≥95.0      |
| (1R)-(−)-Myrtenal | 215.74                     | 98.0       |
| (1R)-(−)-Myrtenol | 224.81                     | 95.0       |
| (−)-(E)-pinocarveol| 217.50                     | ≥96.0      |
| (1S)-(−)-Verbenone| 227.50                     | ≥93.0      |
| Zerumbone         | 321.61                     | ≥98.0      |
| Deet              | 297.45                     | 97.0       |

**Table 6.** Boiling points of 14 pure organic compounds and commercial repellent deet tested for repellency.
half. One side was treated with different amounts (326.2, 163.1, 81.5 and 16.3 μg cm\(^{-2}\)) of each test compound in 1 mL methanol, and the other side was treated with 1 mL methanol. Deet served as the positive control and was used in a similar manner. After drying the samples in a fume hood for 1 min, each treated paper was placed on the bottom section of a disposable Petri dish (15-cm diameter × 2 cm). The lid of the dish had a 1 cm hole cut in the center for introducing the cockroach directly into the center of the arena. One insect at a time was introduced. The hole was then blocked by using a small piece of tissue paper to prevent the cockroaches from escaping. Immediately after the introduction of the insect, the number of seconds it spent on the treated or untreated side within 300 s was timed with two stopwatches. If a compound produced ≥40% repellency at a given concentration, further bioassays were conducted. Filter papers and cockroaches were used once then discarded. Each trial was repeated 10 times.

To determine an effective mixture ratio for ZER and another seven active compounds, the repellency of binary mixtures at five tested ratios (100:0, 70:30, 50:50, 30:70 and 0:100 by weight) was tested at a concentration of 81.5 μg cm\(^{-2}\) at the 24 h post-treatment, which was based on the preliminary test results. Deet served as the positive control. All treatments were replicated 10 times.

**Ebeling choice box assay.** The residual repellency of the eight selected compounds to male *B. germanica* was tested in Ebeling choice boxes as described previously by Appel et al., with a slight modification. A choice test was carried out using a series of two connected acrylic boxes (each 5.5 × 11 × 11 cm). The top side of each compartment of the choice box had nine holes (0.5-mm diameter) on the surface to permit airflow. Except for the top side of the dark compartment of the choice box, the inner surface of other five walls was evenly painted with 652.4 μg cm\(^{-2}\) of the test compounds in 2 mL of methanol using a brush, and the amount was based on the preliminary test results. Food and water were placed on the bottom section of the dark compartment of the choice box. Deet served as a positive control and was similarly formulated. Negative controls (i.e., no test material or repellent) consisted of 2 mL methanol only. Treatments were allocated randomly to the choice boxes. After drying the samples in a fume hood for 3 min, 20 adult male *B. germanica* were released into the untreated compartment of the choice box and were allowed to enter the treated compartment for 24 h. Cockroaches were able to move freely between the dark (treated) and the lighted (untreated) compartments through two holes (1-cm diameter) in the partition separating the sides. Choice boxes had the same environmental conditions as those used for colony maintenance. Banks of white fluorescent lights were 1.6 m above the choice boxes and produced a light intensity in the untreated compartment of 300–350 lux using an INS Digital Lux Meter (Markson Scientific, Phoenix, AZ, USA). The number of *B. germanica* in each compartment was recorded at every 15 min interval until 24 h post-treatment. Six replicates were used for each treatment in a completely randomized design.

For the tests that used antennectomized male *B. germanica*, a razor blade was used to remove the antennae at the scape, as described by Petterson et al. The cockroaches were allowed to recover from the procedure for 24 h before they were exposed to 652.4 μg cm\(^{-2}\) of each compound and deet in 2 mL methanol according to the choice box method above.

**Data analysis.** The repellent index was calculated according to the following formula: % repellency for filter-paper choice assay = \([(Tu - Tt)/Tn] \times 100\), where *Tu* is the number of seconds a cockroach spent on the untreated side, *Tt* is the number of seconds a cockroach spent on the treated side, and *Tn* is the total number of
seconds (300)\textsuperscript{31}; % repellency for the Ebeling choice box assay = 100 – \{[Ta/Tb] × 100\}, where Ta is the number of cockroaches in the treated group and Tb is the number of total cockroaches that were tested\textsuperscript{41}. The percentages of repellency were transformed to arcsine square-root values for analysis of variance (ANOVA). The Bonferroni multiple-comparison method was used to test for significant differences among treatments\textsuperscript{48}. A Student’s t-test was used to test for significant differences between the two treatment methods\textsuperscript{48}. A compound with <10% repellency was considered to be ineffective. Means ± standard errors (SEs) of untransformed data are reported.

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**Acknowledgements**

This work was supported by the National Vector Control and Surveillance Work of the National Institute of Health of the Republic of Korea and by the Brain Korea 21 PLUS program through the National Research Foundation of Korea funded by the Ministry of Education of the Korean Government.

**Author Contributions**

Conceived and designed the experiments: K.-S.C. and Y.-J.A. Performed the experiments: K.-S.C., J.-H.J., G.-H.K., C.-W.C. and S.-J.J. Analyzed the data: K.-S.C., Y.-R.J. and Y.-J.A. Wrote the paper: K.-S.C. and Y.-J.A. Supervised the project: Y.-R.J. and Y.-J.A. Interpreted the spectroscopic data: K.-S.C. and Y.-J.A. Critically revised the manuscript: K.-S.C. and Y.-J.A. Read and approved the final manuscript: K.-S.C., J.-H.J., G.-H.K., C.-W.C., S.-J.J., Y.-R.J. and Y.-J.A.

**Supplementary Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-16099-6.

**Competing Interests:** The authors declare that they have no competing interests.

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