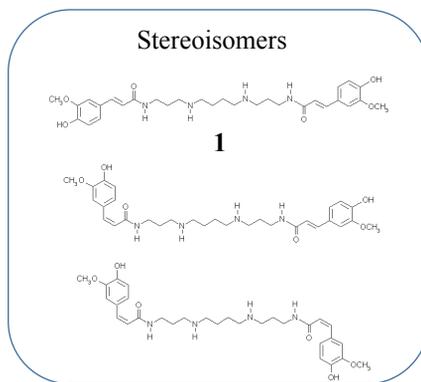
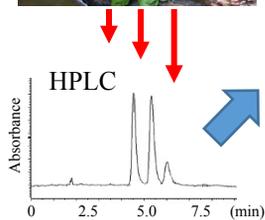




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N^1, N^{14} -diferuloylspermine identified from *Cardamine fauriei*, a wild, edible Brassicaceae herb native to Hokkaido, Japan was found to have high scavenging activity against ROO^\cdot , $O_2^{\cdot-}$ and HO^\cdot radicals.

1 N^1, N^{14} -diferuloylspermine as an antioxidative phytochemical contained in leaves of *Cardamine*

2 *fauriei*

3

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12

13 **ABSTRACT**

14

15 Most Brassicaceae vegetables are ideal dietary sources of antioxidants beneficial for human
16 health. *Cardamine fauriei* (Ezo-wasabi in Japanese) is a wild, edible Brassicaceae herb native to
17 Hokkaido, Japan. To clarify the main antioxidative phytochemical, an 80% methanol extraction
18 from the leaves was fractionated with Diaion[®] HP-20, Sephadex[®] LH-20, and Sep-Pak[®] C18
19 cartridges, and the fraction with strong antioxidant activity depending on DPPH method was
20 purified by HPLC. Based on the analyses using HRESIMS and MS/MS, the compound might
21 be *N*¹,*N*¹⁴-diferuloylspermine. This rare phenol compound was chemically synthesized, whose
22 data on HPLC, MS and ¹H NMR were compared with those of naturally derived compound
23 from *C. fauriei*. All results indicated they were the same compound. The radical-scavenging
24 properties of diferuloylspermine were evaluated by ORAC and ESR spin trapping methods,
25 with the diferuloylspermine showing high scavenging activities of the ROO[·], O₂^{·-} and HO[·]
26 radicals as was those of conventional antioxidants.

27

28 **Keywords:** Brassicaceae; ESR spin trapping; ORAC; Polyamine feruloyl amide; Stereoisomer.

29

30 The increase of oxidative stress in an organism can be the cause of several diseases.
31 Oxidative stress is caused by Reactive Oxygen Species (ROS); including the ROO[·], O₂⁻ and
32 HO[·] radicals.¹⁾ In general, ROS are produced by respiration, photosynthesis and some
33 cell-mediated immune functions.^{2,3)} ROS induced oxidative damage to biomolecules such as
34 lipids, nucleic acids, proteins and carbohydrates can result in ageing, cancer and many other
35 diseases. The HO[·] radical is very reactive among ROS, and leads to damage in cellular
36 components because it can rapidly attack several molecules.⁴⁾ Furthermore, the HO[·] radical leads
37 to lipid peroxidation because it is capable of starting oxidation of polyunsaturated fatty acids.
38 Antioxidants, such as those rich varieties found in fruits and vegetables, edible horticultural
39 products, can protect from ROS.⁵⁾ The consumption of vegetables and fruits can decrease the
40 risk of heart disease and many types of cancer.^{6,7)} Typical dietary antioxidants include ascorbic
41 acid, tocopherols, carotenoids and flavonoids.²⁾ Phenolic compounds, ubiquitous in plants are an
42 essential part of the human diet, and are of considerable interest due to their antioxidant
43 properties.⁸⁾ The Brassicaceae plant is a rich source of these antioxidants.⁹⁾

44 *Cardamine fauriei* Maxim., also known in Japan as Ezo-wasabi, is a plant native to
45 Hokkaido, Japan, and is a perennial Brassicaceae plant. The plant has a unique wasabi-like
46 flavor caused by three glucosinolates,¹⁰⁾ making it a popular edible wild herb in Hokkaido.¹¹⁾
47 Micropropagation technique and hydroponic culture system for the cultivation of the herb had
48 been established.¹²⁾ However, *C. fauriei* is not a commercial vegetable yet. To stimulate interest
49 in cultivating *C. fauriei* as a novel vegetable and as an ingredient of functional foods, it is
50 important to clarify the beneficial antioxidant component contained in the plant. In this study, we
51 examined the *C. fauriei* plant for any antioxidative phytochemicals.

52

53 **Materials and methods**

54 *Reagents.*

55 For the Fractionation process: Diaion[®] HP-20 was purchased from Mitsubishi Chemical
56 (Tokyo, Japan), Sephadex[®] LH-20 from Sigma Aldrich Japan (Tokyo, Japan) and the Sep-Pak[®]
57 C18 cartridges from Waters (Tokyo, Japan). For the chemical synthesis:
58 4-hydroxy-3-methoxycinnamic acid ethyl ester (ethyl ferulate), pyridinium *p*-toluenesulfonate
59 (PPTS), 3,4-dihydro-2*H*-pyran (DHP), *N,N'*-dicyclohexylcarbodiimide (DCC) and
60 *N*-hydroxysuccinimide (NHS) were purchased from Tokyo Chemical Industry (Tokyo, Japan)
61 and spermine from Nacalai Tesque (Kyoto, Japan). For the antioxidant activity analysis:
62 2,2-diphenyl-1-picrylhydrazyl (DPPH) and fluorescein sodium salt were purchased from Sigma
63 Aldrich Japan (Tokyo, Japan), 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH), H₂O₂,
64 diethylenetriaminepentaacetic acid (DTPA), glycine and riboflavin from Wako Pure Chemical
65 (Tokyo, Japan), 2- (5,5-dimethyl-2-oxo-2λ5-[1,3,2] dioxaphosphinan-2-yl)
66 -2-methyl-3,4-dihydro-2*H*-pyrrole 1-oxide (CYPMPO) from Radical Research (Hino, Japan).
67 For the standards: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was
68 purchased from Sigma Aldrich Japan (Tokyo, Japan), ferulic acid, L-ascorbic acid and α -lipoic
69 acid from Wako Pure Chemical (Tokyo, Japan), and quercetin from Kanto Chemical (Tokyo,
70 Japan).

71

72 *Plant material.*

73 The *C. fauriei* plants utilized for this study were hydroponically cultured as described

74 previously.¹²⁾ Mature leaves were frozen with liquid nitrogen and lyophilized. These freeze-dried
75 samples were then powdered.

76

77 *Extraction, fractionation and purification of antioxidative phytochemical.*

78 All fractions were analyzed by DPPH method for screening antioxidative phytochemical, and
79 the highest active fraction was separated by subsequent chromatography. Analysis using this
80 artificial radical was carried out as described previously.¹³⁾ The fractions evaporated and
81 dissolved in 80% EtOH were used as samples for DPPH assay. The 50 μ L of the samples or the
82 standards were added to a 150 μ L solution of DPPH (400 μ M in EtOH):
83 morpholinoethanesulfonic acid (MES) buffer (pH 6.0, 200 mM): 20% EtOH=1:1:1 (v/v/v) into
84 96-well plate. The mixture was left to stand at room temperature for 20 min; then the absorbance
85 was read at 520 nm in a microplate reader (Powerscan HT; DS Pharma Biomedicals, Osaka,
86 Japan). DPPH radical scavenging activity was estimated as the μ mol Trolox equivalent of a
87 sample using the standard curve of Trolox.

88 The lyophilized leaves (14 g) of *C. fauriei* were extracted with 1.0 L of 80% (v/v) MeOH for
89 24 h. The extract was filtrated and evaporated to give crude material, which was subjected to
90 column chromatography using a glass column (500 mm \times 20 mm) packed with Diaion[®] HP-20
91 (50 g) and eluted by a stepwise gradient of water and MeOH. The 80% MeOH fraction was
92 chromatographed using a glass column (500 mm \times 20 mm) packed with Sephadex[®] LH-20 (30
93 g) and eluted with MeOH. The mixed fraction having strong antioxidant activity was further
94 purified by a Sep-Pak[®] C18 cartridges and eluted with a stepwise gradient of water and MeOH.
95 The 50% MeOH fraction was finally purified by HPLC to afford natural compound **1** (0.5mg);

96 ^1H NMR (MeOH- d_4 , 500 MHz) and ^{13}C -NMR (MeOH- d_4 , 125 MHz): see Tables 1 and 2;

97 HRESIMS: m/z 555.3177 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{30}\text{H}_{43}\text{O}_6\text{N}_4$, 555.3183).

98

99 *Synthesis of N^1, N^{14} -diferuloylspermine.*

100 The synthesis of N^1, N^{14} -diferuloylspermine was carried out with some modifications, as
101 described previously.¹⁴⁾

102 *Synthesis of compound 4.* To a stirred solution of compound **2** (5 g, 22.5 mmol) in CH_2Cl_2
103 (70 mL) was added PPTS (630 mg, 2.51 mmol) and DHP (4.4 g, 51.8 mmol), and the reaction
104 mixture was further stirred for 12 h. The usual work up was employed and the resulting material
105 was subjected to silica gel column chromatography (Si 150g, EtOAc: *n*-hexane =2:8) to afford
106 compound **3**. To a stirred mixture of compound **3** in EtOH (40 mL) was added KOH (2.2 g, 39.2
107 mmol), and the reaction mixture was further stirred for 12 h. The usual work up was employed,
108 and the resulting material was subjected to silica gel column chromatography (Si 140g, EtOAc:
109 *n*-hexane =2:8) to compound **4** (2.52 g, 9 mmol, 40% from compound **2**); ^1H NMR (CDCl_3 ,
110 270MHz) δ 7.70 (1H, d, $J=16.0$ Hz, H-7), 7.11-7.06 (3H, m, H-2, H-5 and H-6), 6.29 (1H, d,
111 $J=16.0$ Hz, H-8), 5.46 (1H, m, THP) 3.87 (4H, complex, OCH_3 and THP), 3.61 (1H, m, THP),
112 1.63-2.09 (6H, m, THP); EIMS m/z 278 $[\text{M}]^+$ (3) 194 (100), 85 (37), 41 (21).

113 *Synthesis of compound 5.* To a stirred mixture of compound **4** (2.52 g, 9 mmol) in DMF
114 (20 mL) and THF (60 mL) was added NHS (4.1 g, 36 mmol) and DCC (3.6g, 18 mmol), and
115 the reaction mixture was further stirred for 12 h. The usual work up was employed, and the
116 resulting material was subjected to silica gel column chromatography (Si 150g, EtOAc:
117 *n*-hexane =4:6) to compound **5** (200 mg, 0.5 mmol, 6%); ^1H NMR (CDCl_3 , 270MHz) δ 7.84

118 (1H, d, $J=16.0$ Hz, H-7), 7.16-7.07 (3H, m, H-2, H-5 and H-6), 6.43 (1H, d, $J=16.0$ Hz, H-8),
119 5.47 (1H, m, THP), 3.88 (4H, complex, OCH₃ and THP), 3.60 (1H, m, THP), 2.85 (4H, s, OSu),
120 1.54-2.02 (6H, m, THP); FDMS m/z 377 (3) [M]⁺, 85 (100), 290 (94), 375 (44).

121 *Synthesis of compound 1.* To a stirred mixture of compound **5** (200mg, 0.5 mmol) in
122 CH₂Cl₂ (40 mL) cooled with ice was added compound **6** (54.46 mg, 0.27 mmol), and the
123 reaction mixture was further stirred for 12 h. The resultant mixture was roughly purified to give
124 compound **7**. The protective group of compound **7** was removed using PPTS (100mg, 0.4
125 mmol) in MeOH according to the usual manner. The resultant crude mixture was purified by
126 HPLC, whose condition was mentioned the above, to afford compound **1** (2 mg, 14 μ mol, 3%).

127

128 *HPLC.*

129 The fraction obtained from the Sep-Pak[®] C18 cartridges was separated and purified by
130 HPLC under the following conditions: intelligent pump, L-2160 (Hitachi, Tokyo, Japan);
131 column, Inert Sustain C18 (3 \times 150 mm, GL Sciences, Tokyo, Japan); column temperature,
132 40°C (Model CO631A, GL Sciences, Tokyo, Japan); PDA detector, 280 nm (Model L-2455U,
133 Hitachi, Tokyo, Japan); Auto sampler, L-2200U (Hitachi), flow rate, 0.5 mL min⁻¹; mobile phase,
134 gradient analysis of aq.1.5% formic acid (v/v) and MeOH.

135

136 *HRESIMS, MS/MS, EIMS and FDMS.*

137 The purified fraction from HPLC was analyzed in positive ion mode using a LTQ-Orbitrap
138 XL (ThermoScientific, Waltham, USA) under the following conditions: ionization, electro-spray
139 ionization; m/z , 150 - 2000; spraying voltage, 2.1kV; capillary temp., 200 °C; capillary voltage,

140 40 V; tube lens voltage, 180 V; activation type, collisionally induced dissociation (CID);
141 normalized collision energy, 35%; isolation width, 1.0; activation time, 30 msec. The
142 synthesized compound was analyzed by EIMS and FDMS spectra, and these analysis were
143 recorded with JMS-SX102A (JEOL Tokyo, Japan) and JMS-T 100GCV (JEOL, Tokyo, Japan)
144 spectrometers.

145

146 *NMR*

147 ¹H NMR, ¹³C NMR, ¹H-¹H COSY, HMBC and HSQC were recorded on a Bruker AMX-500
148 or a JEOL JNM EX-270. A sample of approximately 4 mg was dissolved in MeOH-d₄ or CDCl₃
149 and used for recording the spectra. Chemical shift values were expressed in ppm relative to the
150 internal standard, tetramethylsilane.

151

152 *Antioxidant activity for natural ROS.*

153 The synthesized *N*¹,*N*¹⁴-diferuloylspermine (diferuloylspermine) and conventional
154 antioxidants (ferulic acid, quercetin, ascorbic acid and Trolox) were dissolved in MWA
155 (methanol: water: acetic acid = 90:9.5:0.5 (v/v/v)). The activity of scavenging natural ROS
156 (ROO[·], O₂^{·-} and HO[·] radical, respectively) was estimated utilizing the following methods.

157 *ORAC method.* Analysis was carried out according to the previous method.¹⁵⁾ The 35 μL
158 of the synthesized diferuloylspermine, conventional antioxidants, Trolox standards or a blank
159 were added to a 115 μL solution of fluorescein (110.7 mmol/L) and a 50 μL solution of AAPH
160 (31.7 mmol/L) into a 96-well plate. After covering the plate with a film (NJ-500; Takara Bio,
161 Otsu, Japan), the fluorescence intensity (excitation at 485 nm, emission at 530 nm) was

162 monitored at 37 °C every two min for a total of 90 min using a microplate reader. The net area
163 under the curve (AUC) was calculated by subtracting the AUC for the blank from the reagents
164 or standards. The ORAC value was estimated as the μmol Trolox equivalent of a sample using
165 the standard curve of Trolox.

166 *ESR spin trapping method.* Analysis was carried out according to the method as described
167 previously.¹⁶⁻¹⁸⁾ The 50 μL of the synthesized diferuloylspermine, conventional antioxidants, the
168 standards or a blank were added to a 50 μL solution of precursor/sensitizer, a 20 μL solution of
169 CYPMPO (10 mmol/L) and an 80 μL solution of sodium phosphate buffer into an ESR flat cell.
170 In these cases, the precursor/sensitizer reagents utilized for superoxide and hydroxyl radicals
171 were riboflavin and hydrogen peroxide, respectively. The α -lipoic acid and ascorbic acid were
172 used as the standard scavengers for superoxide and hydroxyl radicals, respectively. The ESR flat
173 cell was set in an ESR cavity, and was then irradiated 5 sec with ultraviolet ray for producing
174 radicals. At this time, the ESR spectrum was immediately measured using an X-band ESR
175 spectrometer (JES-RE1X, JEOL, Tokyo, Japan) with a 100 kHz field modulation. The
176 spectrometer conditions were as follows: resonance field, 3521 G; field modulation width, 1.0
177 G; microwave power, 6 mW; light source, 200 W medium pressure mercury/xenon arc lamp
178 (LC-8, Hamamatsu Photonics K.K., Hamamatsu, Japan); UV irradiation intensity for photolysis,
179 2.78 mW/cm² (LC-8, Hamamatsu Photonics K.K., Hamamatsu, Japan) measured by a UV
180 intensity meter (Cole-Parmer International, Illinois, USA); the band-pass filter, G-533 (HOYA,
181 Tokyo, Japan). The analysis of adducted signal was carried out as described by Kameya et al.
182 (2014). The scavenging activities were estimated as the μmol standard equivalent of a sample
183 using the standard curve.

184

185 *Statistical analyses.*

186 Analysis of the antioxidant activity of the synthesized compound and each standard was
187 performed three times independently. Results are shown as an average \pm SE ($n = 3$). Data were
188 analyzed statistically using analysis of variance (ANOVA) and Fisher's F-test followed by
189 Tukey's Multiple Range Test.

190

191 **Results and discussion**

192 *Elucidation of the antioxidative phytochemical in C. fauriei.*

193 The DPPH method has been used popularly for screening antioxidant potential of both
194 individual phenolics and biologically relevant samples like foods,¹⁹⁾ and for measuring radical
195 scavenging capacity of pure compounds, food constituents, plant extracts and the other samples
196 such as synthesized compounds²⁾. Therefore, we employed the DPPH method for screening
197 antioxidative compounds. Using several purification steps (Fig. S1), a most active ingredient,
198 compound **1** (0.5 mg, Fig. 1), having antioxidant property was purified from lyophilized leaves
199 (14 g) of *C. fauriei*. Although the compound **1** was once purified, re-chromatogram of
200 compound **1** using HPLC gave three major peaks in HPLC feature (Fig. 2).

201 The purified compound showed the accurate mass values at m/z 555.3177 and the molecular
202 formula was estimated to be $C_{30}H_{43}O_6N_4 [M+H]^+$. In the ESIMS/MS spectra, typical fragments
203 were observed at m/z 177, 234 and 305, which are distinctive for diferuloylspermine residues.²⁰⁾
204 Furthermore, the 1H NMR spectrum showed resonances of aromatic protons at 6.75 - 7.34 ppm,
205 methyl proton at 3.87 ppm and methylene in spermine part at 1.73 - 3.41 ppm. Therefore, it was

206 hypothesized that natural compound might be N^1, N^{14} -diferuloylsperimine. To confirm the
207 hypothesis, the N^1, N^{14} -diferuloylsperimine was chemically synthesized according to the reported
208 method with some modifications (Fig. S2).¹⁴⁾ The $^1\text{H-NMR}$ (Fig. 3A) and MS data of
209 synthesized compound showed well coincidence with those of naturally obtained
210 diferuloylsperimine, compound **1**. Therefore, the chemical structure of the isolated natural
211 compound **1** was determined to be N^1, N^{14} -diferuloylsperimine.

212 It has been generally accepted that (*E*) geometry of olefin in α, β unsaturated carbonyl moiety
213 should be more stable than (*Z*) one, although these are interchangeable, and we had guessed that
214 the synthesized compound should have (*E*) geometry of olefin due to use of compound **2** as a
215 starting material. However, the $^1\text{H NMR}$ spectrum of synthesized compound **1** was complicated
216 as same as that of naturally derived N^1, N^{14} -diferuloylsperimine (**1**). Therefore, it was determined
217 that some parts of (*E*) geometry of olefin were interconverted to (*Z*) configuration when the
218 coupling reaction of **5** and **6** or leaving the protection group for phenolic hydroxyl moiety in **7**
219 was performed. The cross peak in HMBC between δ 170.1 ($-\underline{\text{C}}\text{ONH-}$) / δ 6.43 ($-\text{CH}\beta=\underline{\text{C}}\text{H}\alpha-$, $J=$
220 15.5 Hz, Fig. 3B) and 3.41 (H-2) were observed, which established the ^{13}C and ^1H assignments
221 of (*E*) form part together with the information of HSQC which confirmed direct connectivity
222 between H and C and $^1\text{H-}^1\text{H}$ COSY which indicated the connectivity of H-2/H-3/H-4 and
223 H-6/H-7. Applying the same strategy of building up the connectivity using the information of
224 cross peaks between δ 171.0 ($-\underline{\text{C}}\text{ONH-}$) / δ 5.85 ($-\text{CH}\beta=\underline{\text{C}}\text{H}\alpha-$, $J=$ 12.5 Hz, Fig. 3B) and 3.34
225 (H-2) in HMBC spectra together pursuing the cross peaks in HSQC and $^1\text{H-}^1\text{H}$ COSY spectra,
226 the ^{13}C and ^1H assignments of (*Z*) form moiety were determined. Since we could not find the
227 cross peak between δ 2.97 (H-4 and 6 of *E* form) and 46.6 (C-4 and 6 of *Z* form) nor δ 2.88 (H-4

228 and 6 of *Z* form) and 46.4 (C-4 and 6 of *E* form), we established above total assignments. It
229 might be the possibility that the assignments of H-6 and C-6 were δ 2.97 and 46.4 in (*Z*) form
230 and δ 2.88 and 46.6 in (*E*) geometry. This was the reason why we put the interchangeable
231 possibility in Tables 1 and 2. We could not determine for abundance ratio of the (*E, E*), (*E, Z*),
232 and (*Z, Z*) stereoisomers of the synthesized and naturally obtained compound **1** at room
233 temperature. But, we reached conclusion that each (*E, E*), (*E, Z*), and (*Z, Z*) stereoisomer existed
234 because re-chromatogram of purified naturally obtained and synthesized compound **1** gave three
235 peaks around 5 min having the accurate mass values at m/z 555.3177 in HRESIMS analysis (Fig.
236 2). Therefore, data of Tables should be considered to be resulted from mixture of (*E, E*), (*E, Z*),
237 (*Z, Z*) forms (Figs. S3 and S4). To clarify the abundance ratio of the isomers, it should be
238 necessary to analyze prior to change into the stereoisomers such as using HPLC connecting with
239 NMR. Finally, the total assignments of ^1H and ^{13}C NMR of N^1, N^{14} -diferuloylsperimine (**1**) for
240 (*E*) and (*Z*) forms were firstly given in this paper (Tables 1 and 2).

241 Above mentioned confirmation, (*E*) and (*Z*) mixture, was agreed with the previous
242 reports,²¹⁻²³⁾ in which they reported that the complexity of the NMR spectrum of
243 hydroxycinnamic acid spermidines was attributed to the mixture of *E-Z* configurational isomers.
244 Furthermore, it was also found that the isomers (*E* or *Z* form) of spermidine conjugate changed
245 dramatically and rapidly upon exposure to sunlight and irradiation by UV.^{22, 23)} Based on our
246 synthetic experiment, it was firstly reconfirmed that diferuloylsperimine can be easily converted
247 into (*E, E*), (*E, Z*), and (*Z, Z*) stereoisomers.

248

249 *Antioxidant activity of diferuloylsperimine.*

250 Although diferuloylspermine was found to be an antioxidative phytochemical for DPPH
251 artificial radical in this study, it was not clear if this compound could have scavenging activity
252 for naturally occurred ROS. So, the antioxidant activity of diferuloylspermine was evaluated by
253 the ORAC (ROO[·] radical) and ESR spin trapping (O₂^{·-} and HO[·] radicals) methods, then
254 compared with those of conventional antioxidants: quercetin, ferulic acid, ascorbic acid and
255 Trolox (Fig. 4). In this case, the content of DFSM (1.01 μmol·g DW⁻¹) in *C. fauriei* leaves was
256 greater than that of quercetin (0.12 μmol·g DW⁻¹), a major antioxidant in *Brassica* vegetables.²⁴⁾
257 With the ORAC (ROO[·] radical) and ESR spin trapping (O₂^{·-} radical) assay, the radical
258 scavenging activities of diferuloylspermine were the same or larger than those of conventional
259 antioxidants except for quercetin with the highest values. These results supported the previous
260 fact that quercetin showed higher scavenging activities than Trolox for both ROO[·] and O₂^{·-}
261 radical.⁵⁾ In addition, with the ESR spin trapping (HO[·] radical) assay, diferuloylspermine showed
262 the highest activity among antioxidants examined. It has been considered that ferulic acid and
263 ascorbic acid are powerful antioxidants.^{2, 25, 26)} Furthermore, flavonoids including quercetin is
264 recognized to be antioxidants, and they have high scavenging activity for HO[·] radical.^{2, 27)} Since
265 diferuloylspermine showed the same scavenging activities for ROO[·] and O₂^{·-} radicals as ferulic
266 acid and ascorbic acid, and the highest scavenging activity for HO[·] radical, it might be that
267 diferuloylspermine has a good potential of natural antioxidant correspond to conventional
268 antioxidants. It was reported that N¹,N⁵,N¹⁰-Tris (4-hydroxycinnamoyl) spermidine showed
269 scavenging activity for DPPH radical, whereas analog hydroxyl groups which were methylated
270 exhibited a very weak activity.²⁸⁾ This report also indicated that the scavenging activity was
271 caused by presence of the phenolic OH groups of hydroxycinnamoyl moieties. Furthermore,

272 spermine was reported to exhibit HO[·] radical scavenging activity.²⁹⁾ Therefore, it seemed that the
273 high HO[·] radical scavenging activity of diferuloylspermine was due to OH groups of ferulic acid
274 and spermine moiety.

275 As physiological function of diferuloylspermine, *N*¹,*N*¹⁴ - bis (dihydrocaffeoyl) spermine,
276 Kukoamine A, was isolated from the root bark of *Lycium chinese* as clinically effective
277 hypotensive compound.³⁰⁾ Furthermore, Kukoamine A and the analogs showed
278 anti-trypanosomal activity due to inhibit of trypanothione reductase.³¹⁾ Since the structure of
279 diferuloylspermine is analogs to Kukoamine A, this compound might also have those
280 physiological functions. Previously, diferuloylspermine was found only in the reproductive
281 organs of *Ananas comosms*, *Gomphrena globose* and *Zea mays*.^{32,33)} This is the first report on
282 diferuloylspermine isolated from *Brassicaceae* plants, especially not from reproductive organs
283 but vegetative parts of the plant.

284

285 **Author contributions**

286

287 Study concept and design: Takashi Suzuki. Acquisition of data: Keima Abe, Hideyuki
288 Matsuura, and Mitsuko Ukai. Analysis and interpretation of data: Keima Abe, Hideyuki
289 Matsuura, Mitsuko Ukai, Hanako Shimura, Hiroyuki Koshino, and Takashi Suzuki. Drafting of
290 the manuscript: Keima Abe, Hideyuki Matsuura, and Takashi Suzuki. All authors reviewed and
291 approved the final manuscript.

292

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294

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301

302 **Disclosure statement**

303

304 No potential conflict of interests was reported by the authors.

305

306 **Supplemental material**

307

308 Supplemental material for this article can be accessed at doi.

309

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391

392 **Legend of figures**

393

394 Fig. 1. Chemical structure of compound **1** (*E*)-form.

395

396 Fig. 2. HPLC features of re-chromatogram of naturally obtained and synthesized compound **1**.

397

398 Fig. 3. ¹H-NMR analysis of naturally obtained and synthesized compound **1**.

399 Notes: A: ¹H-NMR spectrum of natural derived and synthesized compound **1**; B:

400 Aromatic/olefinic region of the ¹H NMR spectrum of synthesized compound **1** showing signal

401 (MeOH-d₄), and *J* values of the olefin units.

402

403 Fig. 4. Antioxidant activity about synthesized diferuloylspermine and antioxidant standards.

404 Notes: Graphs of each radical scavenging activity are shown: A: ROO[·]; B: O₂^{·-}; C: HO[·].

405 Equivalent activity is shown: ROO[·] radical, Trolox; O₂^{·-} radical, α- Lipoic acid; HO[·] radical,

406 Ascorbic acid. Values are means ± SE of three independent experiments. Different letters at the

407 top of bars indicate significant differences between standards (*P* < 0.05).

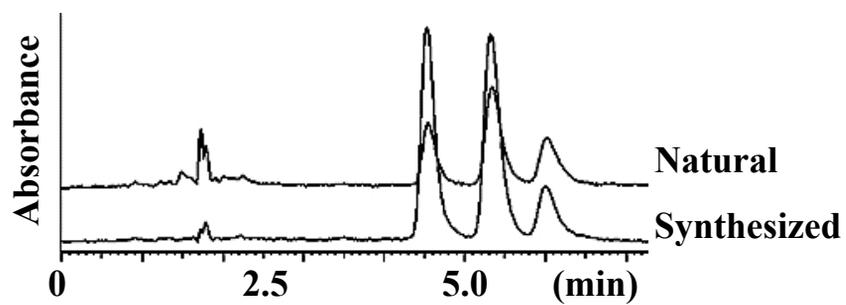
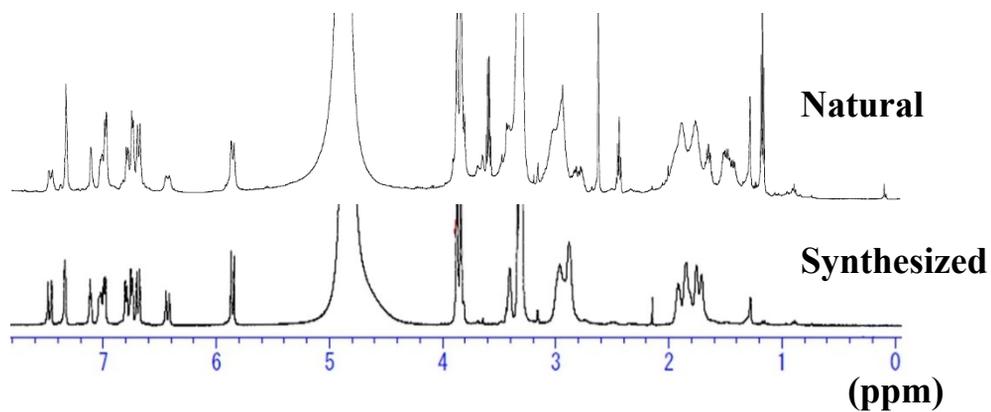


Fig. 2

A



B

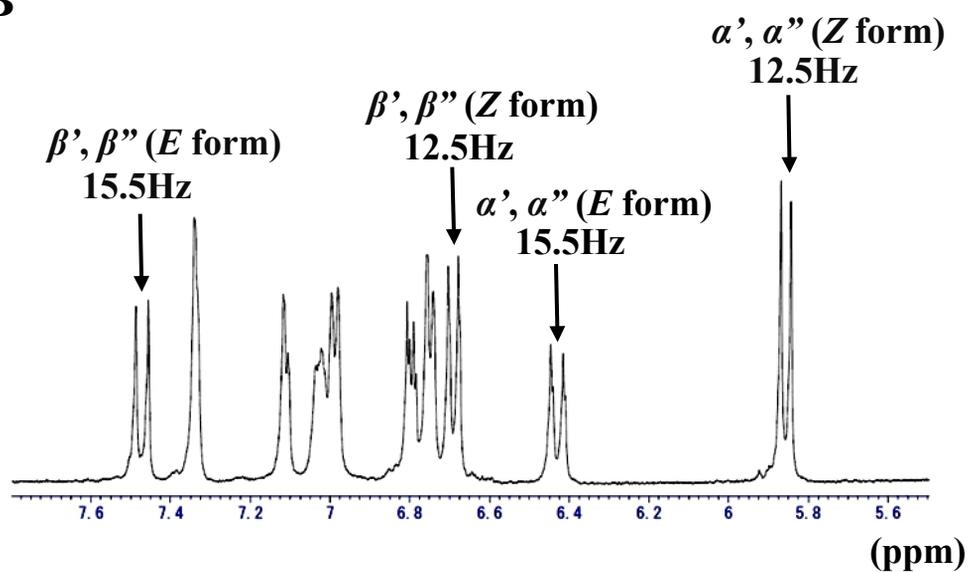


Fig. 3

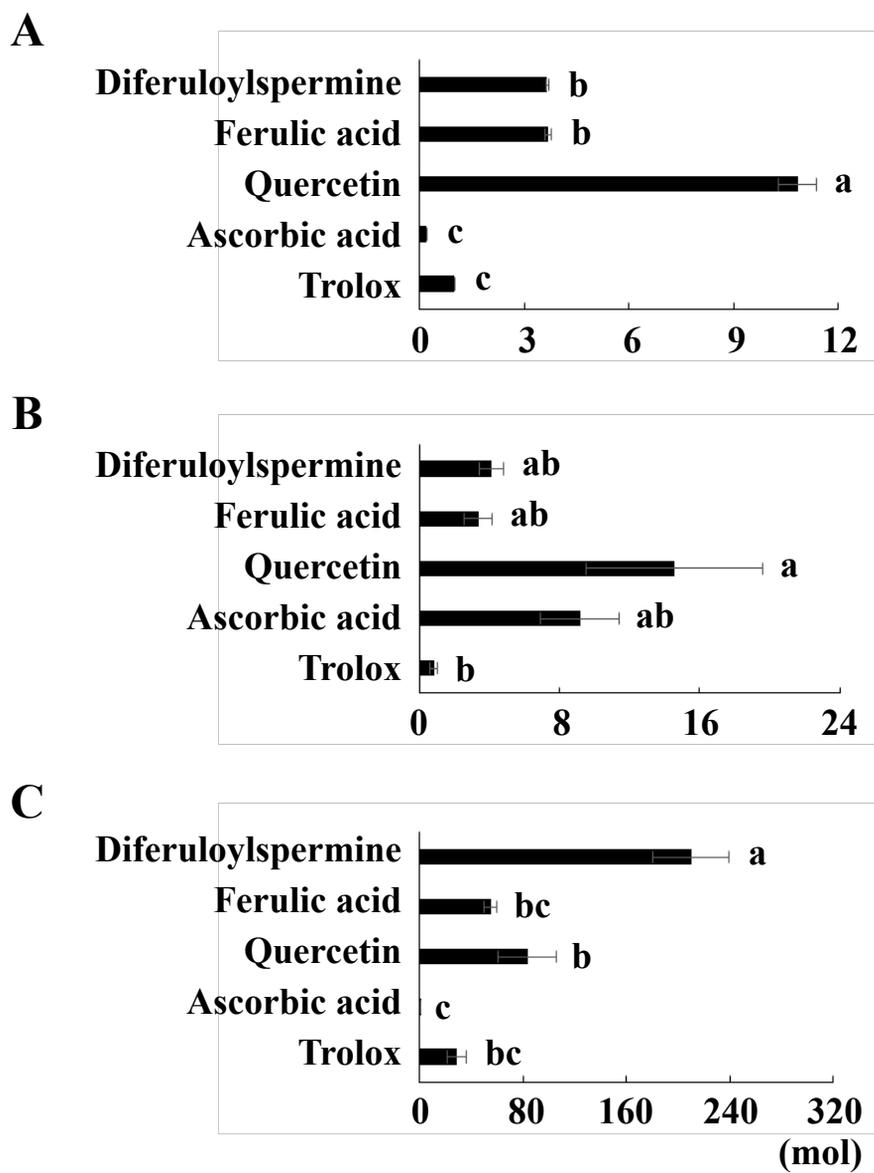


Fig. 4

Table 1. ^1H - and ^{13}C -NMR Data ^{a)} of Compound **1** (*E* form) in MeOH- d_4 .

Position	δ_{C}	δ_{H} (<i>J</i> in Hz)	HMBC ^{b)}
2	37.2	3.41, m	3, -CONH-
3	28.3	1.92, m	2
4	46.4	2.97, m	2,3
6	46.4 ^{c)}	2.97 ^{d)} , m	
7	25.3	1.73, m	
1'	128.0		5', α' , β'
2'	111.6	7.12, m	6', β'
3'	149.4		5', OCH ₃
4'	150.2		2', 6'
5'	116.6	6.81, m	
6'	123.5	7.02, m	2', β'
α'	118.1	6.43, d (15.5)	β' , -CONH-
β'	142.8	7.47, d (15.5)	2', 6'
CONH	170.1		2, α' , β'
OCH ₃	56.5	3.87, s	

a) The resonances were assigned for (*E*) part of (*E, E*) and (*E, Z*) mixtures.

b) HMBC correlations are from proton (s) stated to the indicated carbon.

c) Interchangeable to 46.6.

d) Interchangeable to 2.88.

Table 2. ^1H - and ^{13}C -NMR Data ^{a)} of Compound **1** (*Z* form) in MeOH-d_4 .

Position	δ_{C}	δ_{H} (<i>J</i> in Hz)	HMBC ^{b)}
2	37.2	3.34, m	3, -CONH-
3	28.0	1.85, m	2
4	46.6	2.88, m	2,3
6	46.6 ^{c)}	2.88 ^{d)} , m	
7	25.3	1.73, m	
1'	130.0		5', α'
2'	114.4	7.34, m	6', β'
3'	148.6		2',5', OCH_3
4'	148.6		2',5',6'
5'	116.0	6.75, m	
6'	124.8	6.98, m	2', β'
α'	121.4	5.85, d (12.5)	β' , -CONH-
β'	139.3	6.69, d (12.5)	2',6', α'
CONH	171.0		2, α' , β'
OCH_3	56.5	3.87, s	

a) The resonances were assigned for (*Z*) part of (*Z*, *Z*) and (*E*, *Z*) mixtures.

b) HMBC correlations are from proton (s) stated to the indicated carbon.

c) Interchangeable to 46.4.

d) Interchangeable to 2.97.

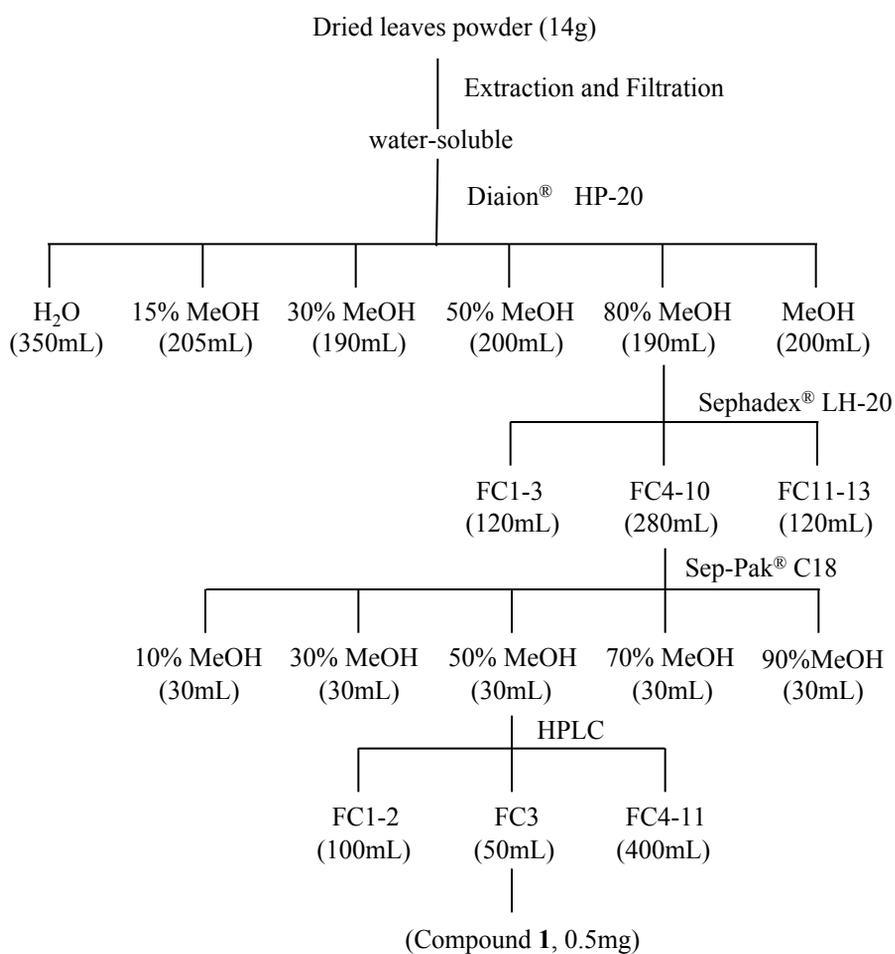


Fig. S1. Purification procedure for the isolation of an antioxidative phytochemical, compound **1**, from dried leaf blade powder of *C. fauriei*.

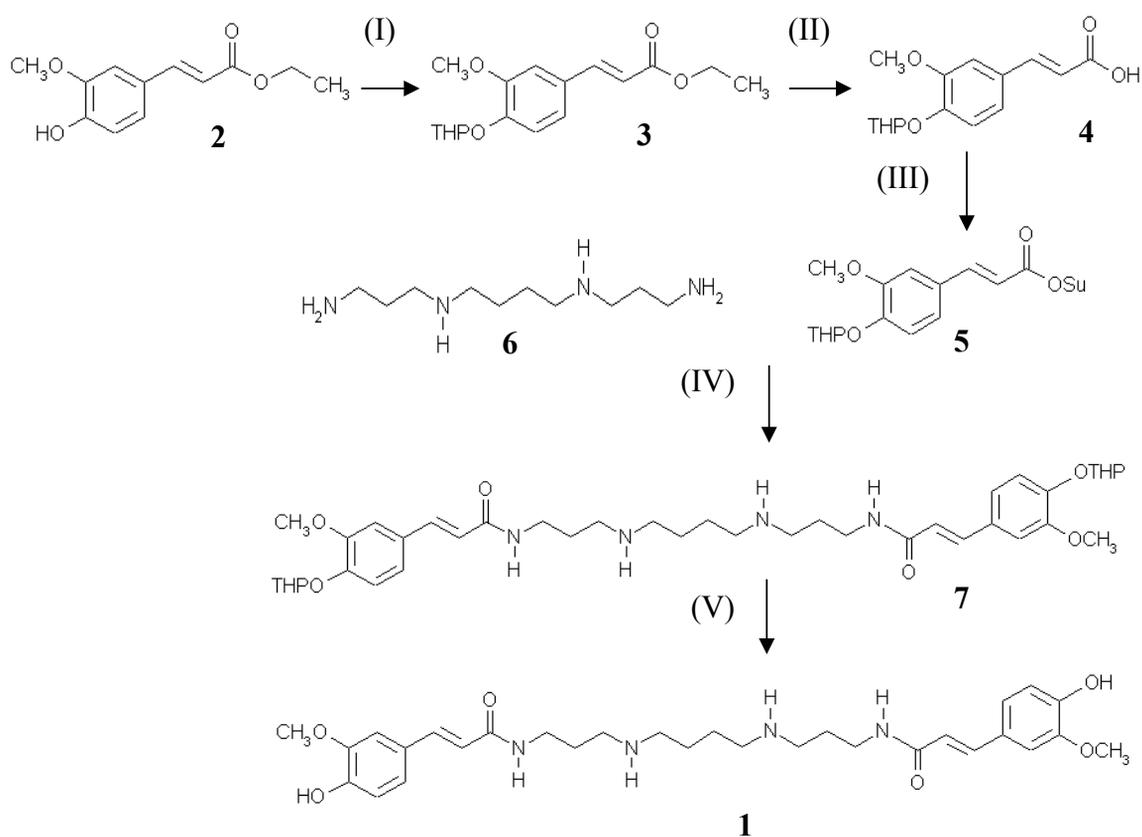


Fig. S2. Synthesis procedure of N^1, N^{14} -diferuloylspermine. (I) DHP, PPTS, CH_2Cl_2 ; (II) KOH, EtOH (40%, over two steps); (III) DCC, NHS, DMF, THF (6%); (IV) Spermine (**6**), CH_2Cl_2 ; (V) PPTS, MeOH (3%, over two steps).

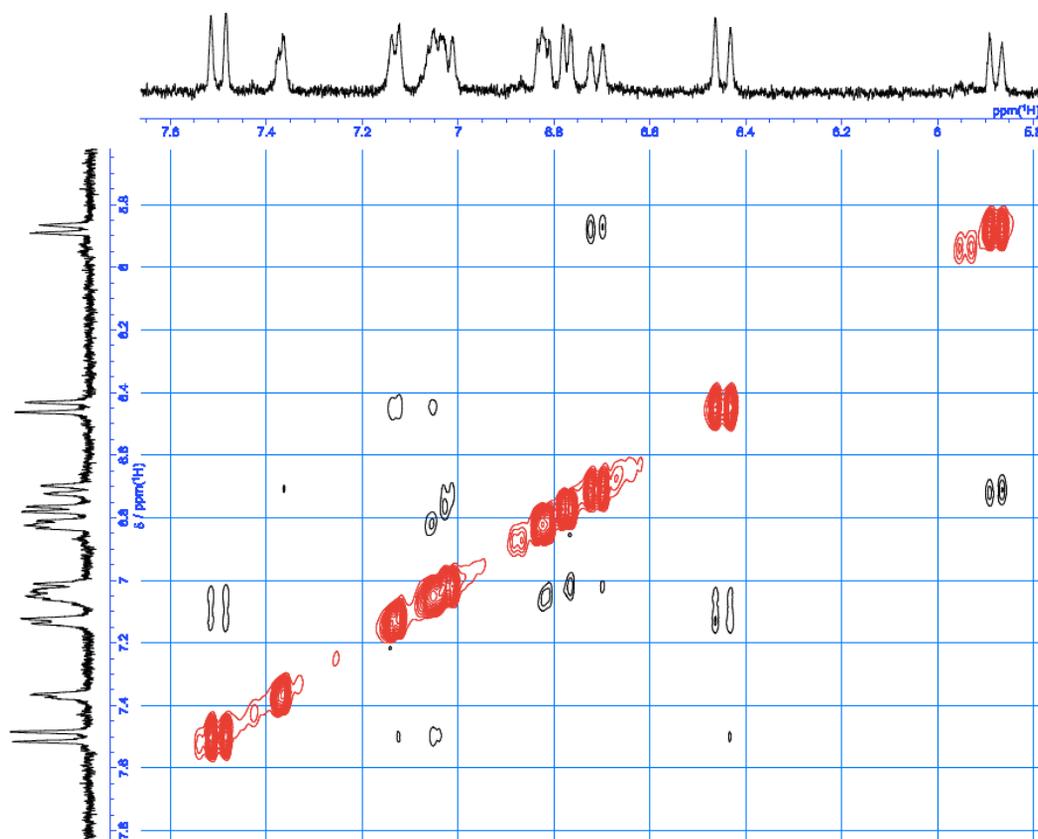


Fig. S3. NOESY Spectra of N^1, N^{14} -diferuloylspermine.

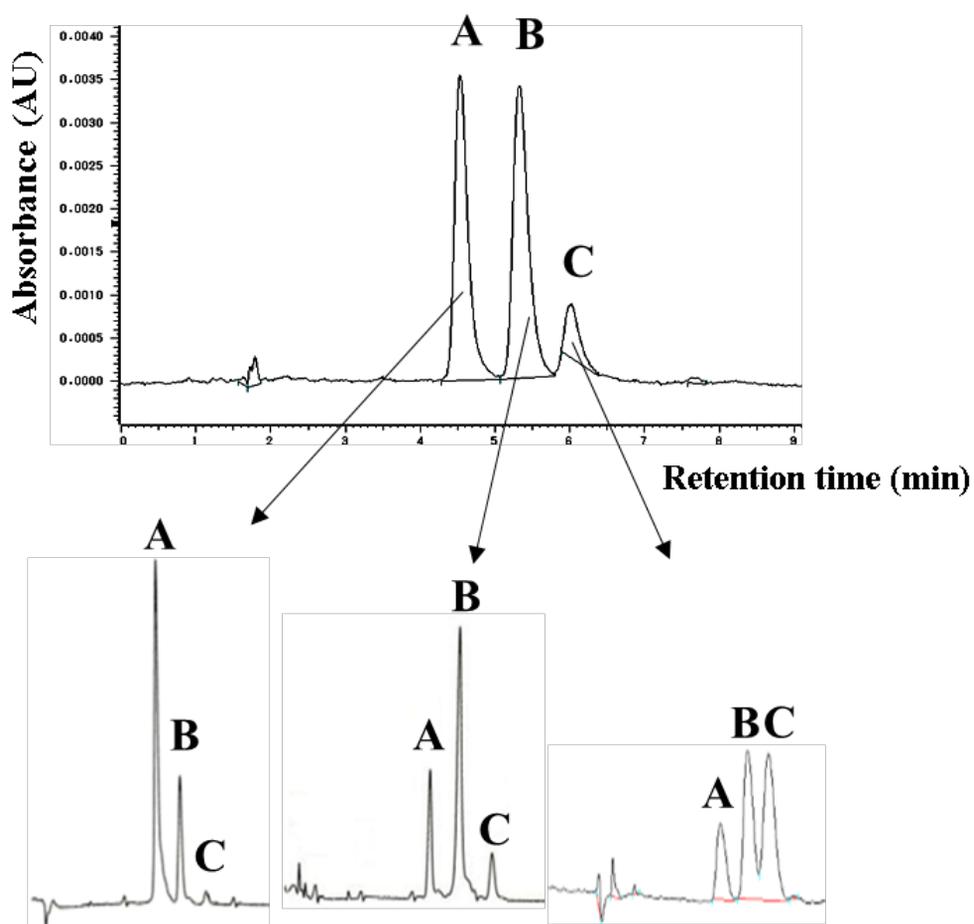


Fig. S4. Re-chromatograms of N^1, N^{14} -diferuloylspermine.