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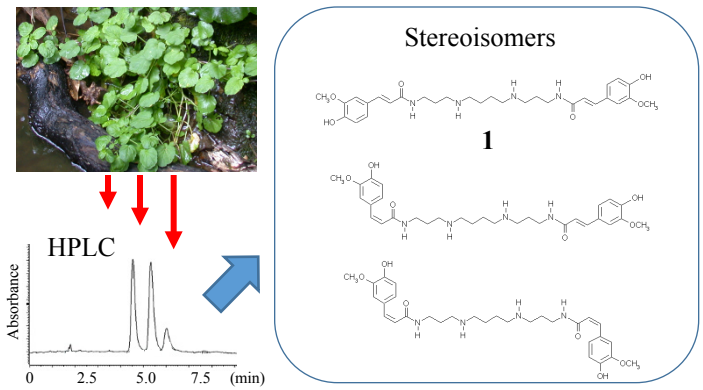

N^1,N^{14}-diferuloylspermine identified from *Cardamine fauriei*, a wild, edible Brassicaceae herb native to Hokkaido, Japan was found to have high scavenging activity against $ROO^{\cdot}$, $O_2^{\cdot-}$ and $HO^{\cdot}$ radicals.

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

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ABSTRACT

Most Brassicaceae vegetables are ideal dietary sources of antioxidants beneficial for human health. *Cardamine fauriei* (Ezo-wasabi in Japanese) is a wild, edible Brassicaceae herb native to Hokkaido, Japan. To clarify the main antioxidative phytochemical, an 80% methanol extraction from the leaves was fractionated with Diaion® HP-20, Sephadex® LH-20, and Sep-Pak® C18 cartridges, and the fraction with strong antioxidant activity depending on DPPH method was purified by HPLC. Based on the analyses using HRESIMS and MS/MS, the compound might be N^1,N^{14}-diferuloylspermine. This rare phenol compound was chemically synthesized, whose data on HPLC, MS and ^{1}H NMR were compared with those of naturally derived compound from *C. fauriei*. All results indicated they were the same compound. The radical-scavenging properties of diferuloylspermine were evaluated by ORAC and ESR spin trapping methods, with the diferuloylspermine showing high scavenging activities of the $ROO^{\cdot}$, $O_2^{\cdot-}$ and $HO^{\cdot}$ radicals as was those of conventional antioxidants.

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

The increase of oxidative stress in an organism can be the cause of several diseases. Oxidative stress is caused by Reactive Oxygen Species (ROS); including the $ROO^{\cdot}$, $O_2^{\cdot -}$ and $HO^{\cdot}$ radicals.[1] In general, ROS are produced by respiration, photosynthesis and some cell-mediated immune functions.[2, 3] ROS induced oxidative damage to biomolecules such as lipids, nucleic acids, proteins and carbohydrates can result in ageing, cancer and many other diseases. The $HO^{\cdot}$ radical is very reactive among ROS, and leads to damage in cellular components because it can rapidly attack several molecules.[4] Furthermore, the $HO^{\cdot}$ radical leads to lipid peroxidation because it is capable of starting oxidation of polyunsaturated fatty acids. Antioxidants, such as those rich varieties found in fruits and vegetables, edible horticultural products, can protect from ROS.[5] The consumption of vegetables and fruits can decrease the risk of heart disease and many types of cancer.[6, 7] Typical dietary antioxidants include ascorbic acid, tocopherols, carotenoids and flavonoids.[2] Phenolic compounds, ubiquitous in plants are an essential part of the human diet, and are of considerable interest due to their antioxidant properties.[8] The Brassicaseae plant is a rich source of these antioxidants.[9]

Cardamine fauriei Maxim., also known in Japan as Ezo-wasabi, is a plant native to Hokkaido, Japan, and is a perennial Brassicaceae plant. The plant has a unique wasabi-like flavor caused by three glucosinolates,[10] making it a popular edible wild herb in Hokkaido.[11] Micropropagation technique and hydroponic culture system for the cultivation of the herb had been established.[12] However, *C. fauriei* is not a commercial vegetable yet. To stimulate interest in cultivating *C. fauriei* as a novel vegetable and as an ingredient of functional foods, it is important to clarify the beneficial antioxidant component contained in the plant. In this study, we 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), H_2O_2,
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 μL of the samples or the
82 standards were added to a 150 μL solution of DPPH (400 μM 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 μmol 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 × 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 × 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);

^{1}H NMR (MeOH-d_4, 500 MHz) and ^{13}C-NMR (MeOH-d_4, 125 MHz): see Tables 1 and 2; HRESIMS: *m/z* 555.3177 $[M+H]^+$ (calcd for $C_{30}H_{43}O_6N_4$, 555.3183).

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

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

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

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

(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*=16.0Hz, H-8), 5.47 (1H, m, THP), 3.88 (4H, complex, OCH_3 and THP), 3.60 (1H, m, THP), 2.85 (4H, s, OSu), 1.54-2.02 (6H, m, THP); FDMS *m/z* 377 (3) $[M]^+$, 85 (100), 290 (94), 375 (44).

*Synthesis of compound **1**.* To a stirred mixture of compound **5** (200mg, 0.5 mmol) in CH_2Cl_2 (40 mL) cooled with ice was added compound **6** (54.46 mg, 0.27 mmol), and the reaction mixture was further stirred for 12 h. The resultant mixture was roughly purified to give compound **7**. The protective group of compound **7** was removed using PPTS (100mg, 0.4 mmol) in MeOH according to the usual manner. The resultant crude mixture was purified by HPLC, whose condition was mentioned the above, to afford compound **1** (2 mg, 14 μmol, 3%).

HPLC.

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

HRESIMS, MS/MS, EIMS and FDMS.

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

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

NMR

^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HMBC and HSQC were recorded on a Bruker AMX-500 or a JEOL JNM EX-270. A sample of approximately 4 mg was dissolved in MeOH-d_4 or $CDCl_3$ and used for recording the spectra. Chemical shift values were expressed in ppm relative to the internal standard, tetramethylsilane.

Antioxidant activity for natural ROS.

The synthesized N^1,N^{14}-diferuloylspermine (diferuloylspermine) and conventional antioxidants (ferulic acid, quercetin, ascorbic acid and Trolox) were dissolved in MWA (methanol: water: acetic acid = 90:9.5:0.5 (v/v/v)). The activity of scavenging natural ROS ($ROO^{\cdot}$, $O_2^{\cdot-}$ and $HO^{\cdot}$ radical, respectively) was estimated utilizing the following methods.

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

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

ESR spin trapping method. Analysis was carried out according to the method as described previously.[16-18] The 50 μL of the synthesized diferuloylspermine, conventional antioxidants, the standards or a blank were added to a 50 μL solution of precursor/sensitizer, a 20 μL solution of CYPMPO (10 mmol/L) and an 80 μL solution of sodium phosphate buffer into an ESR flat cell. In these cases, the precursor/sensitizer reagents utilized for superoxide and hydroxyl radicals were riboflavin and hydrogen peroxide, respectively. The α-lipoic acid and ascorbic acid were used as the standard scavengers for superoxide and hydroxyl radicals, respectively. The ESR flat cell was set in an ESR cavity, and was then irradiated 5 sec with ultraviolet ray for producing radicals. At this time, the ESR spectrum was immediately measured using an X-band ESR spectrometer (JES-RE1X, JEOL, Tokyo, Japan) with a 100 kHz field modulation. The spectrometer conditions were as follows: resonance field, 3521 G; field modulation width, 1.0 G; microwave power, 6 mW; light source, 200 W medium pressure mercury/xenon arc lamp (LC-8, Hamamatsu Photonics K.K., Hamamatsu, Japan); UV irradiation intensity for photolysis, 2.78 mW/cm^2 (LC-8, Hamamatsu Photonics K.K., Hamamatsu, Japan) measured by a UV intensity meter (Cole-Parmer International, Illinois, USA); the band-pass filter, G-533 (HOYA, Tokyo, Japan). The analysis of adducted signal was carried out as described by Kameya et al. (2014). The scavenging activities were estimated as the μmol standard equivalent of a sample using the standard curve.

Statistical analyses.

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

Results and discussion

Elucidation of the antioxidative phytochemical in C. fauriei.

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

The purified compound showed the accurate mass values at *m/z* 555.3177 and the molecular formula was estimated to be $C_{30}H_{43}O_6N_4$ $[M+H]^+$. In the ESIMS/MS spectra, typical fragments were observed at *m/z* 177, 234 and 305, which are distinctive for diferuloylspermine residues.[20] Furthermore, the ^1H NMR spectrum showed resonances of aromatic protons at 6.75 - 7.34 ppm, 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}- diferuloylspermine was chemically synthesized according to the reported
208 method with some modifications (Fig. S2).[14] The ^{1}H-NMR (Fig. 3A) and MS data of
209 synthesized compound showed well coincidence with those of naturally obtained
210 diferuloylspermine, 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}H NMR spectrum of synthesized compound **1** was complicated
216 as same as that of naturally derived N^1,N^{14}- diferuloylspermine (**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 (-CONH-) /δ 6.43 (-CHβ=CHα-, J=
220 15.5 Hz, Fig. 3B) and 3.41 (H-2) were observed, which established the ^{13}C and ^{1}H assignments
221 of (*E*) form part together with the information of HSQC which confirmed direct connectivity
222 between H and C and ^{1}H-^{1}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 (-CONH-)/δ 5.85 (-CHβ=CHα-, J= 12.5 Hz, Fig. 3B) and 3.34
225 (H-2) in HMBC spectra together pursuing the cross peaks in HSQC and ^{1}H-^{1}H COSY spectra,
226 the ^{13}C and ^{1}H 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 ^{1}H 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 diferuloylspermine can be easily converted
247 into (*E, E*), (*E*, *Z*), and (*Z*, *Z*) stereoisomers.
248
249 *Antioxidant activity of diferuloylspermine.*

Although diferuloylspermine was found to be an antioxidative phytochemical for $DPPH^{\cdot}$ artificial radical in this study, it was not clear if this compound could have scavenging activity for naturally occurred ROS. So, the antioxidant activity of diferuloylspermine was evaluated by the ORAC ($ROO^{\cdot}$ radical) and ESR spin trapping ($O_2^{\cdot -}$ and $HO^{\cdot}$ radicals) methods, then compared with those of conventional antioxidants: quercetin, ferulic acid, ascorbic acid and Trolox (Fig. 4). In this case, the content of DFSM (1.01 μmol·g DW^{-1}) in *C. fauriei* leaves was greater than that of quercetin (0.12 μmol·g DW^{-1}), a major antioxidant in *Brassica* vegetables.[24] With the ORAC ($ROO^{\cdot}$ radical) and ESR spin trapping ($O_2^{\cdot -}$ radical) assay, the radical scavenging activities of diferuloylspermine were the same or larger than those of conventional antioxidants except for quercetin with the highest values. These results supported the previous fact that quercetin showed higher scavenging activities than Trolox for both $ROO^{\cdot}$ and $O_2^{\cdot -}$ radical.[5] In addition, with the ESR spin trapping ($HO^{\cdot}$ radical) assay, diferuloylspermine showed the highest activity among antioxidants examined. It has been considered that ferulic acid and ascorbic acid are powerful antioxidants.[2, 25, 26] Furthermore, flavonoids including quercetin is recognized to be antioxidants, and they have high scavenging activity for $HO^{\cdot}$ radical.[2, 27] Since diferuloylspermine showed the same scavenging activities for $ROO^{\cdot}$ and $O_2^{\cdot -}$ radicals as ferulic acid and ascorbic acid, and the highest scavenging activity for $HO^{\cdot}$ radical, it might be that diferuloylspermine has a good potential of natural antioxidant correspond to conventional antioxidants. It was reported that N^1,N^5,N^{10}-Tris (4-hydroxycinnamoyl) spermidine showed scavenging activity for DPPH radical, whereas analog hydroxyl groups which were methylated exhibited a very weak activity.[28] This report also indicated that the scavenging activity was caused by presence of the phenolic OH groups of hydroxycinnamoyl moieties. Furthermore,

spermine was reported to exhibit $HO^{\cdot}$ radical scavenging activity.[29] Therefore, it seemed that the high $HO^{\cdot}$ radical scavenging activity of diferuloylspermine was due to OH groups of ferulic acid and spermine moiety.

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

Author contributions

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

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Disclosure statement

No potential conflict of interests was reported by the authors.

Supplemental material

Supplemental material for this article can be accessed at doi.

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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. ^{1}H-NMR analysis of naturally obtained and synthesized compound **1**.

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

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

401 (MeOH-d_4), 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: $ROO^{\cdot}$; B: $O_2^{\cdot -}$; C: $HO^{\cdot}$.

405 Equivalent activity is shown: $ROO^{\cdot}$ radical, Trolox; $O_2^{\cdot -}$ radical, α- Lipoic acid; $HO^{\cdot}$ 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 ($P < 0.05$).

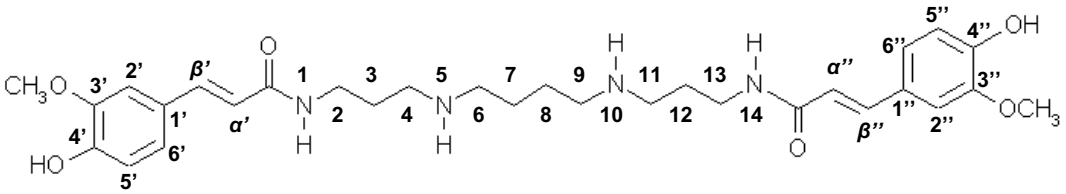
CH3O
HO
OH
OCH3
O
H
N
1
2
3
4
5
6
7
8
9
10
11
12
13
14
1'
2'
3'
4'
5'
6'
α'
β'
1''
2''
3''
4''
5''
6''
α''
β''

Fig. 1

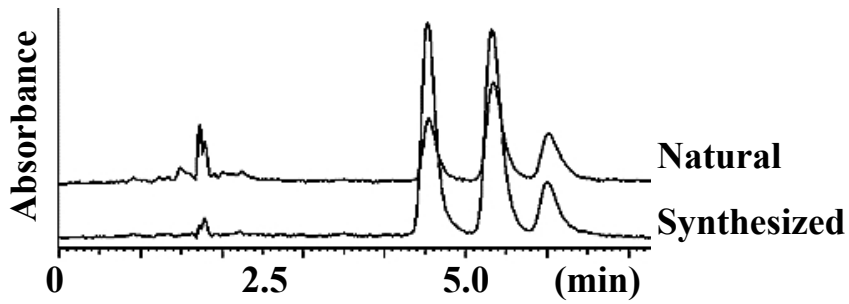

Fig. 2

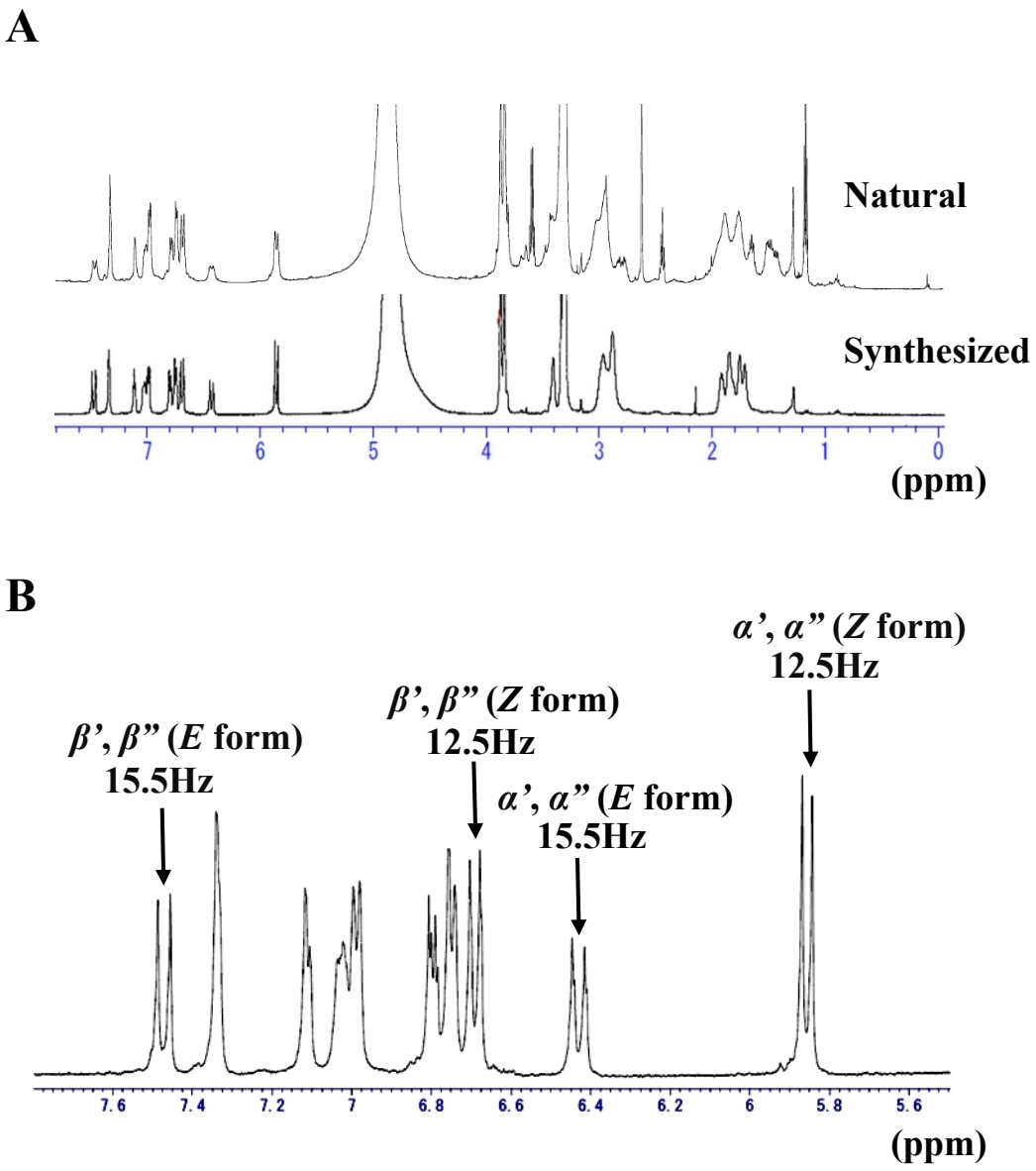
A
Natural
Synthesized
7
6
5
4
3
2
1
0
(ppm)
B
β', β" (E form)
15.5Hz
β', β" (Z form)
12.5Hz
α', α" (E form)
15.5Hz
α', α" (Z form)
12.5Hz
7.6
7.4
7.2
7
6.8
6.6
6.4
6.2
6
5.8
5.6
(ppm)

Fig. 3

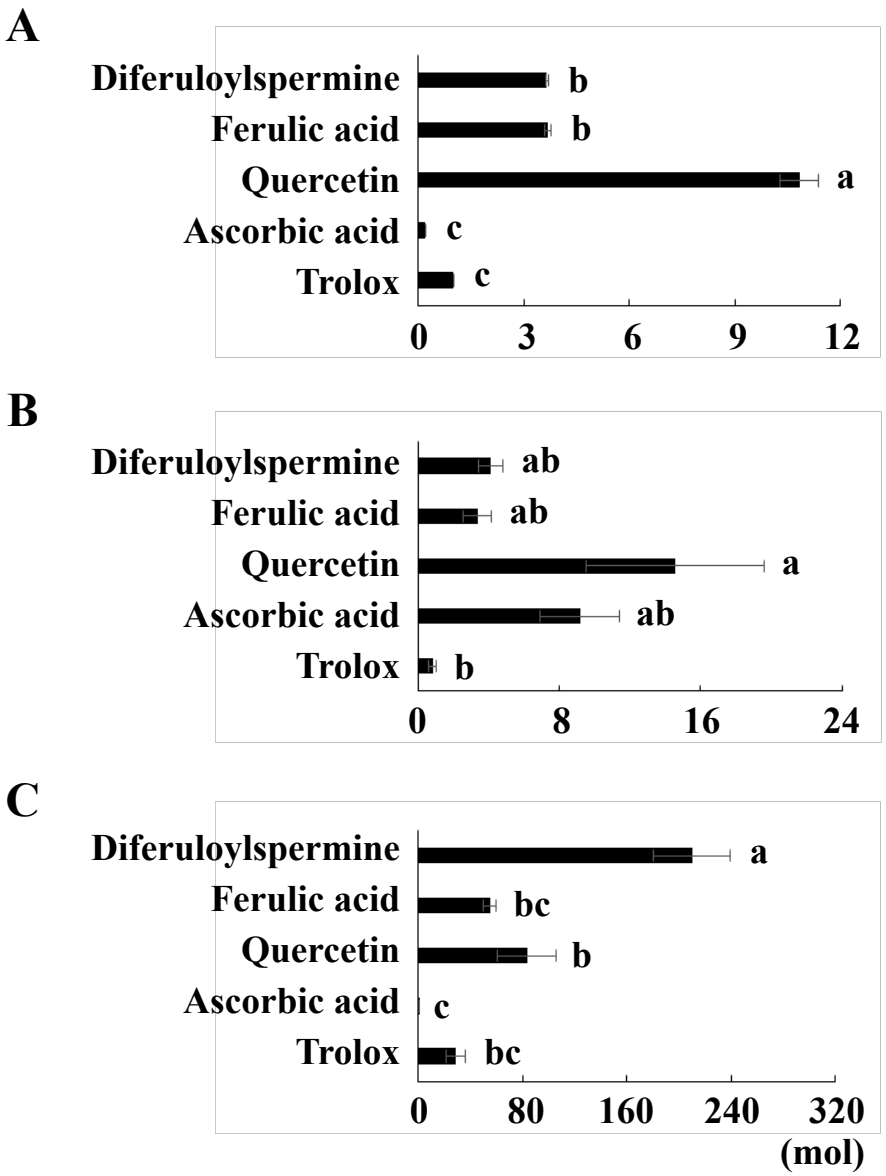
A
Diferuloylspermine
Ferulic acid
Quercetin
Ascorbic acid
Trolox
b
b
a
c
c
0
3
6
9
12
B
Diferuloylspermine
Ferulic acid
Quercetin
Ascorbic acid
Trolox
ab
ab
a
ab
b
0
8
16
24
C
Diferuloylspermine
Ferulic acid
Quercetin
Ascorbic acid
Trolox
a
bc
b
c
bc
0
80
160
240
320
(mol)

Fig. 4

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

Position	δ_C	δ_H (*J* 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) Interchangable to 2.88.

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

Position	δ_C	δ_H (*J* 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) Interchangable to 2.97.

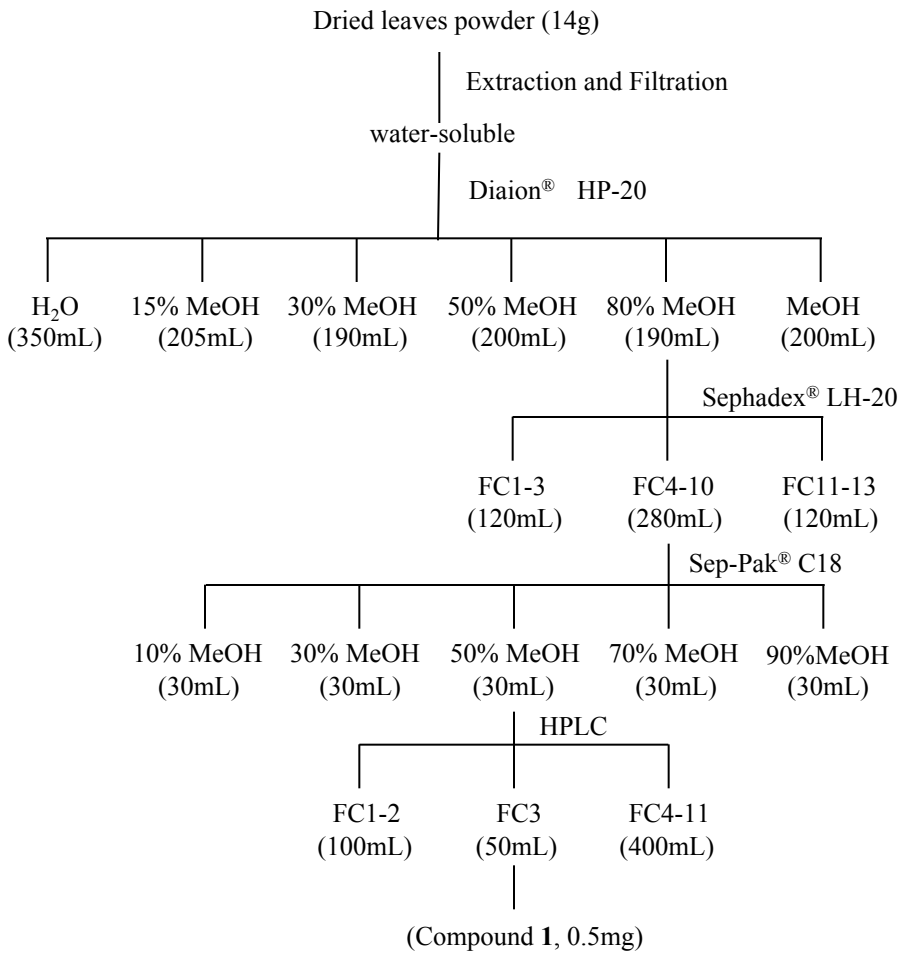

Fig. S1. Purification procedure for the isolation of an antioxidative phytochemical, compound **1**, from dried leaf blade powder of *C. fauriei*.

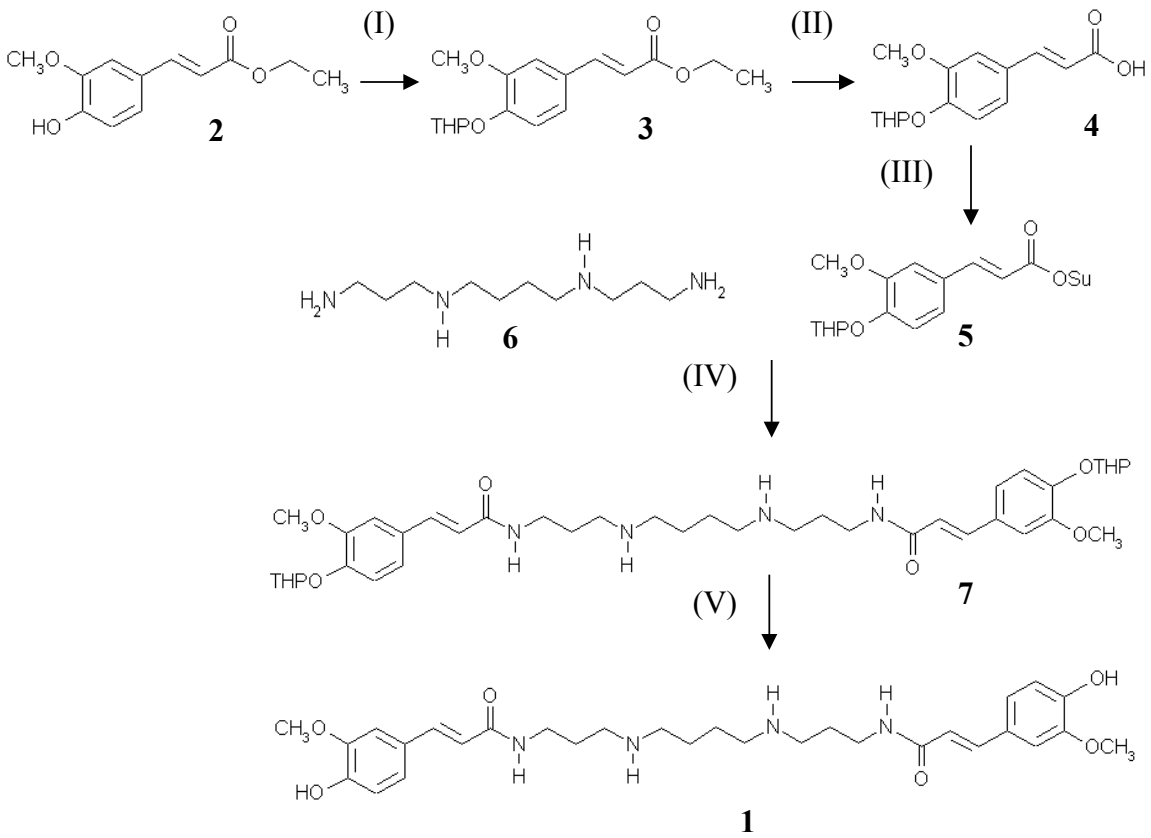

Fig. S2. Synthesis procedure of N^1,N^{14}-diferuloylspermine. (I) DHP, PPTS, CH_2Cl_2; (II) KOH, EtOH (40%, over two steps); (III) DCC, NHS, DMF, THF (6%); (IV) Spermine (**6**), CH_2Cl_2; (V) PPTS, MeOH (3%, over two steps).

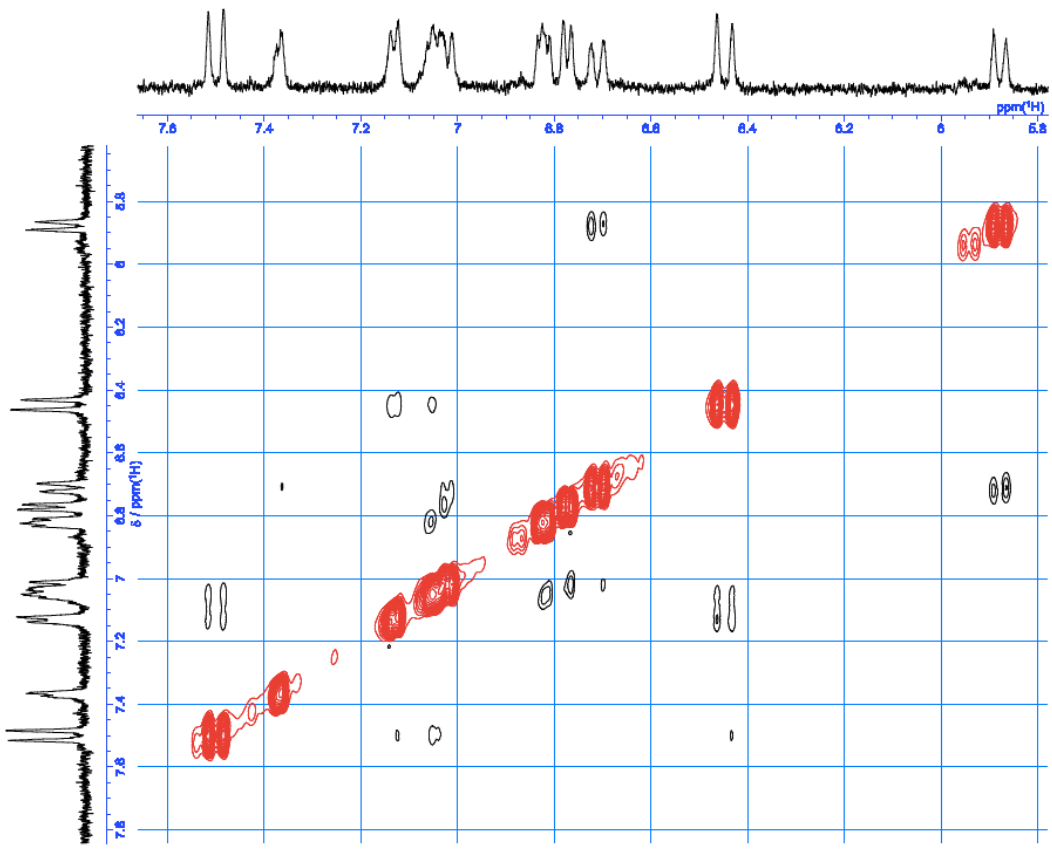

Fig. S3. NOESY Spectra of N^1,N^{14}-diferuloylspermine.

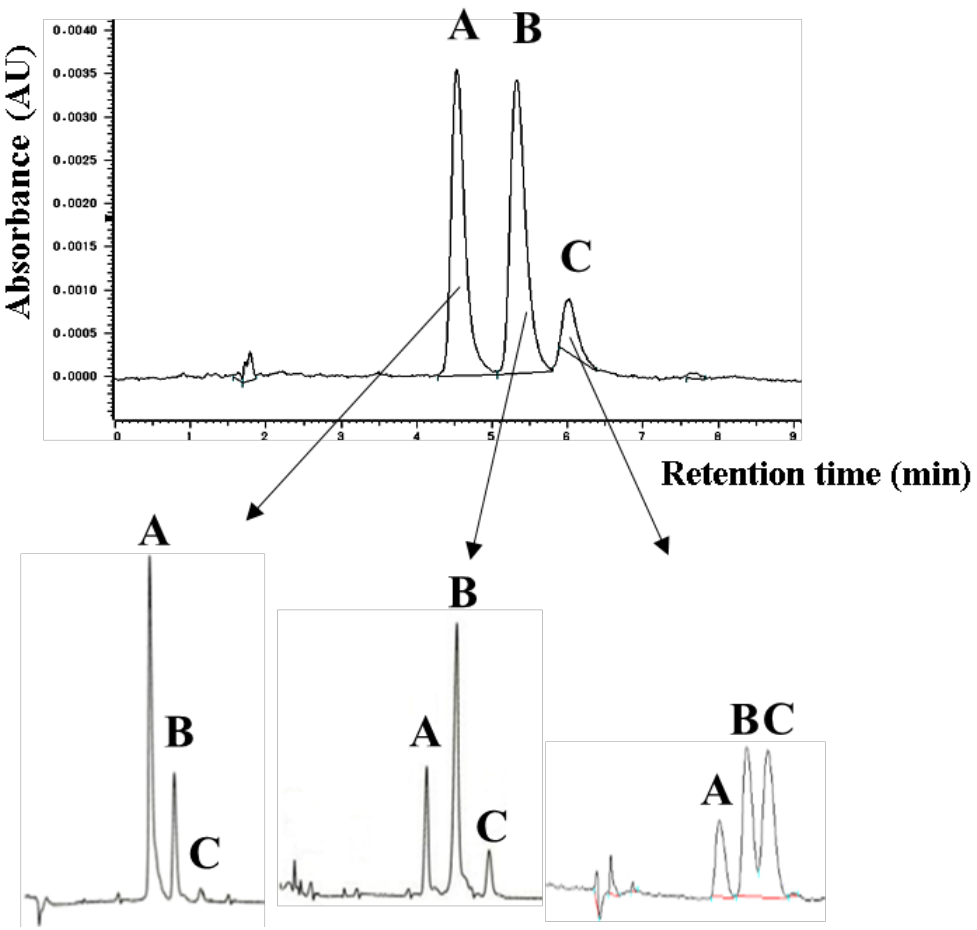

Fig. S4. Re-chromatograms of N^1,N^{14}-diferuloylspermine.