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Top-down Targeted Metabolomics Reveals a Sulfur-Containing Metabolite with Inhibitory Activity against Angiotensin-Converting Enzyme in *Asparagus officinalis*

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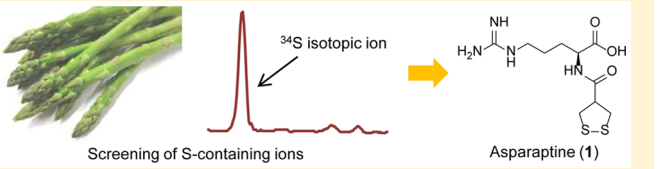
Supporting Information

ABSTRACT: The discovery of bioactive natural compounds containing sulfur, which is crucial for inhibitory activity against angiotensin-converting enzyme (ACE), is a challenging task in metabolomics. Herein, a new S-containing metabolite, asparaptine (**1**), was discovered in the spears of *Asparagus officinalis* by targeted metabolomics using mass spectrometry for S-containing metabolites. The contribution ratio (2.2%) to the IC_{50} value in the crude extract showed that asparaptine (**1**) is a new ACE inhibitor.



Sulfur-containing metabolites (S-metabolites) are common across the plant kingdom and constitute a family of natural products with a wide range of beneficial biological activities for humans, such as anti-inflammatory, antioxidative, and anti-cancer effects. The presence of a sulfur atom is crucial for the inhibitory activity against angiotensin-converting enzyme (ACE), which catalyzes the reaction from angiotensin I to angiotensin II in the rennin−angiotensin system and plays a major role in hypertension.[1,2] Interestingly, a saturated five-membered sulfide ring (1,2-dithiolane ring) in synthetic peptides has a prominent role in the inhibitory activity of ACE.[3]

The inhibitory activity of plant extracts against ACE is broadly investigated.[4] *Asparagus officinalis* L. (Asparagaceae) has a positive effect in the prevention of hypertension by inhibiting the activity of ACE.[5] Reports show that N-containing metabolites (e.g., nicotianamine and 2″-hydroxynicotianamine) are possible inhibitors in asparagus; however, the existence of other molecules has been suggested. Asparagus is compared with *Allium* species (Alliaceae),[6] which accumulate a variety of S-metabolites.[7,8] Therefore, it is hypothesized that asparagus might contain unknown S-metabolites exhibiting inhibitory activity against ACE.

The exact mass and natural abundance of stable isotopes in Nature are reflected in their mass spectrometry (MS) spectra. Sulfur has two major stable isotopes, ^{32}S and ^{34}S, representing their natural abundances of 95.02% and 4.21%, respectively. The theoretical differences in the exact mass and relative signal intensity between ^{32}S-containing monoisotopic ions (S-ions) and their ^{34}S-substituted counterparts can be used for the chemical assignment of S-ions. Since the clear separation of ^{34}S-ions and other ions substituted by ^{18}O and $^{13}C_2$ at the region of the monoisotope (M) + 2 requires high mass accuracy (<1 mDa) and peak resolution [ca. 250 000 full width at half-maximum (fwhm)] in small molecules,[9] these ions have not been used for chemical assignment. Recently, a metabolomic approach based on the targeted analysis for S-metabolites (S-omics) has been established using Fourier transform ion cyclotron resonance mass spectrometry (FTICRMS), which offers the best performance with respect to ultrahigh mass accuracy (<1 ppm) and peak resolution (>1 000 000 fwhm).[10] S-Omics using edible parts of three crops, i.e., onion (*Allium cepa* L.), green onion (*Allium fistulosum* L.), and garlic (*Allium sativum* L.), revealed the distribution of S-metabolites.[11] The tagged information on S-metabolites by FTICRMS coupled with liquid chromatography (LC) may be expected to streamline the isolation and chemical assignment of S-metabolites.

S-Omics was used to seek a S-ion from a new S-metabolite, asparaptine (**1**), which is a conjugate of arginine and asparagusic acid, in 47 plant samples, including asparagus (Table S1, Supporting Information). Aqueous methanol extract solutions of the plants were analyzed using LC−FTICRMS to understand the S-ion distribution in all detectable metabolite peaks. On the basis of the differences in the exact mass and relative signal intensity of S-ions and their ^{34}S-substituted counterparts, 733 putative S-ions were extracted from the metabolome matrix, which included 4537 peaks. Then, the S-ions were filtered using their relative signal intensity value (>1.0) to isolate asparaptine (**1**). Ultimately, elemental composition was assigned for 13 S-ions using their exact mass and isotope pattern (Figure 1A and Table S2, Supporting Information). The S-ion derived from asparaptine (**1**) had the highest relative signal intensity in asparagus. The isotopic pattern was nearly identical to the theoretical one of $C_{10}H_{19}N_4O_3S_2$ (Figure 1B).

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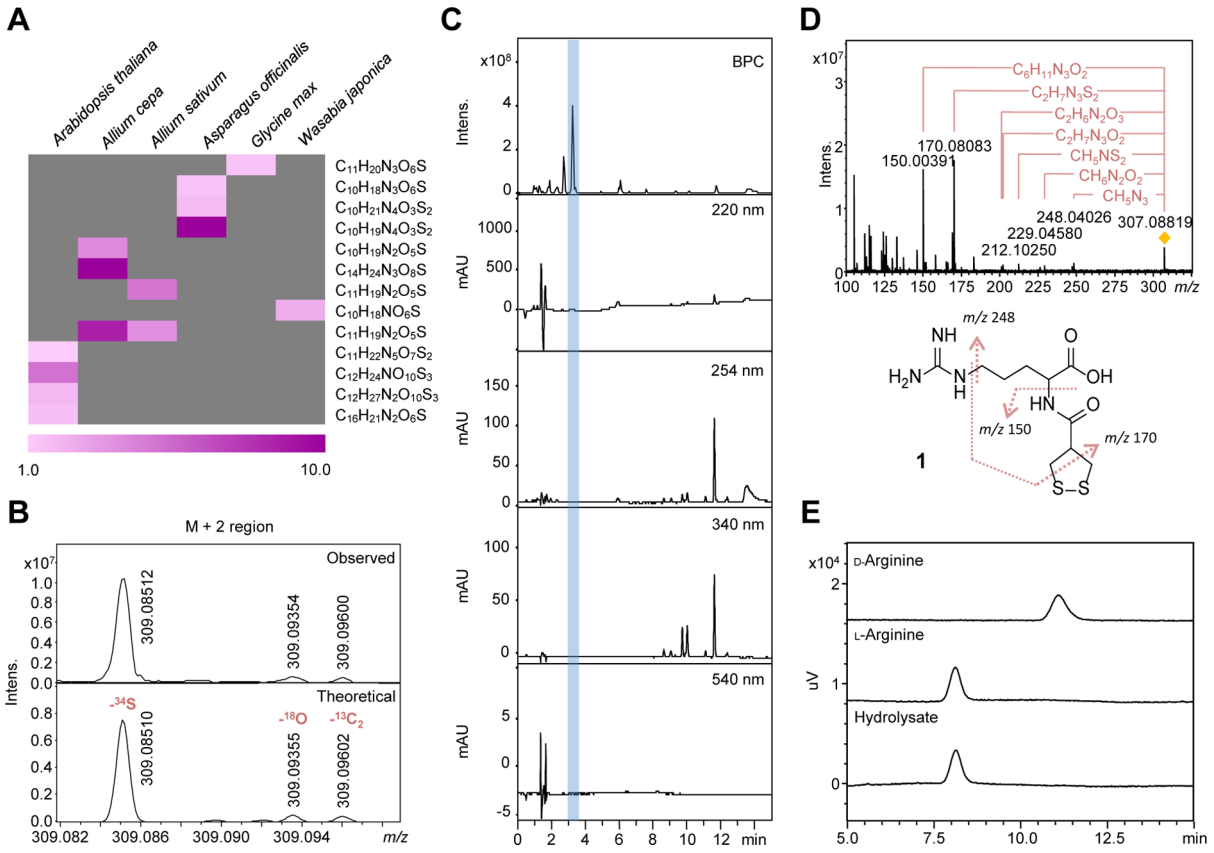
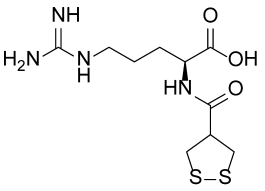


Figure 1. Targeted metabolomics for S-containing metabolites to elucidate the new compound asparaptine (**1**) in *Asparagus officinalis*. (A) Hierarchical cluster analysis of S-containing monoisotopic ions (S-ions). The elemental compositions of S-ions were determined using their exact mass and isotope pattern. The color bar indicates the strength of the relative signal intensity. The gray color indicates less than 1.0 or not detected. (B) Isotope pattern at the region of the monoisotope (M) + 2. The upper diagram indicates ^{34}S-, ^{18}O-, and $^{13}C_2$-substituted ions of the monoisotopic ion (*m*/*z* 307.08931). The lower diagram shows the theoretical pattern of $C_{10}H_{19}N_4O_3S_2$. The resolution power of these ions was approximately 400 000 fwhm. (C) MS- and UV-based chromatograms. The peak range of asparaptine (**1**) is colored in blue. BPC, base peak chromatogram. (D) Product and difference ion analysis. The diamond symbol indicates the protonated molecular ion. (E) Chiral high-performance liquid chromatography analysis of the hydrolysate of asparaptine (**1**) and D,L-arginine. The hydrolysate was identified as L-arginine.



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Subsequently, tandem MS (MS/MS) analysis was performed for chemically assigning the structure of S-metabolites according to the guidelines of the Metabolomics Standards Initiative.[12] Using elemental compositions assigned by the isotope analysis, corresponding S-metabolites were searched in the in-house, ReSpect, and KNApSAcK databases.[13,14] The analysis of product and difference ions (between protonated molecular and product ions) characterized and annotated eight S-ions from known S-metabolites in the databases, such as glucosinolates, glutathione derivatives, and *S*-alkenylcysteines in *Arabidopsis thaliana* L. Heynh, onion, garlic, *Glycine max* L. Merr., and *Wasabia japonica* (Miq.) Matsum. (Table S2 and Figure S1, Supporting Information). However, the structure of three ions in asparagus could not be assigned in this analysis.

The isolation of asparaptine (**1**) was performed using standard chromatographic approaches, and the MS- and UV-based chromatograms were analyzed to elucidate its structure (Figure 1C). The peak of asparaptine (**1**) ($[M + H]^+$, *m*/*z* 307.0893) was confirmed in a base peak chromatogram (BPC), while there were no redundant visible peaks of asparaptine (**1**) in the UV-based chromatograms at 220, 254, 340, and 540 nm. These findings suggested that the structure of asparaptine (**1**) contains no chromophores. Therefore, asparaptine (**1**) was isolated by preparative LC−PDA−MS after open column chromatography. By tracking the ion with *m*/*z* 307 in the positive ion mode, asparaptine (**1**) (61.0 mg) was finally isolated from lyophilized asparagus powder (57.5 g dry weight).

Asparaptine (**1**) was obtained as a white powder, and its elemental composition was determined by FTICRMS to be $C_{10}H_{18}N_4O_3S_2$. By MS/MS analysis, the product ion (*m*/*z* 248.040, $C_9H_{14}NO_3S_2$) and the difference ion (*m*/*z* 59.047, CH_5N_3) suggested the desorption of the guanidine moiety (Figure 1D). The product ion (*m*/*z* 150.003, $C_4H_8NOS_2$) and the difference ion (*m*/*z* 157.084, $C_6H_{11}N_3O_2$) suggested the presence of arginine and asparagusic acid moieties. The ^{1}H NMR, COSY, and HMQC spectra (Figures S2, S3, and S4, Supporting Information) indicated 12 proton signals for 10 protons on five methylene carbons and two methine protons, suggesting that the molecule contains arginine and asparagusic acid moieties. The ^{13}C NMR spectrum (Figure S2, Supporting Information) showed 10 carbon signals for five methylenes (δ 27.2, 31.5, 43.3, 44.6, 45.4), two methines (δ 54.0, 57.6), and

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three quaternary carbons (δ 159.5, 176.7, 181.2). In the HMBC spectrum, the α-proton signal of arginine at δ 4.21 (H-8) revealed a strong correlation with the signal of the amide carbonyl carbon at δ 176.7 (C-6) (Figure S5, Supporting Information). The acid hydrolysate was detected at the same retention time of L-arginine by chiral high-performance liquid chromatography (HPLC) (Figure 1E). Consequently, the chirality of C-8 was determined to be *S*.

Asparaptine (**1**) was assayed to evaluate its inhibitory activity against ACE. Captopril, nicotianamine, and *N*-succinyl-L-proline were used as positive controls, because they possess potent inhibitory activity against ACE. L-Arginine, D-arginine, L-aspartic acid, and asparagusic acid glucose ester[15] were investigated to gain an in-depth insight into the structure–activity relationship. Consequently, the IC_{50} values of captopril, nicotianamine, *N*-succinyl-L-proline, asparaptine (**1**), and asparagusic acid glucose ester were 1.6 nM, 18.7 μM, 14.5 μM, 113 μM, and 347 μM, respectively (Table 1). The inhibitory activity of asparaptine (**1**) was 6-fold lower than that of nicotianamine, for which the inhibitory activity was nearly equal to that of 2″-hydroxynicotianamine.[16] The IC_{50} values of L-arginine, D-arginine, and L-aspartic acid could not be determined. These results suggested that the conjugation of the asparaptine (**1**) or asparagusic acid moiety alone is a key aspect of the inhibitory activity. Next, asparagusic acid was assayed to evaluate its contribution to the inhibitory activity. The IC_{50} value of asparagusic acid could not be determined (Table 1), indicating that the 1,2-dithiolane ring in conjugated form is important for the inhibitory activity. Arginine is more suitable than glucose as the counterpart of the asparagusic acid moiety in the conjugate to exhibit the activity.

Table 1. Compound ACE Inhibitory Activity

compound	IC_{50} (μM)
captopril[a]	0.001 61 ± 0.000 43
N-succinyl-L-proline[a]	14.5 ± 5.3
nicotianamine[a]	18.7 ± 0.54
asparaptine (**1**)[a]	113 ± 11
asparagusic acid glucose ester[a]	347 ± 22
D-arginine[a]	N.D.[c]
L-arginine[a]	N.D.[c]
L-aspartic acid[a]	N.D.[c]
asparagusic acid[b]	N.D.[c]

[a]Compound was dissolved in water. [b]Compound was dissolved in 1% dimethyl sulfoxide. [c]N.D., not determined.

The contribution ratio of asparaptine (**1**) to the ACE inhibitory activity was calculated in the edible parts of green asparagus spears. The IC_{50} value of aqueous methanol extracts of the green asparagus spears was 157 μg/mL. Quantitative analysis using liquid chromatography–quadrupole time-of-flight–mass spectrometry (LC–QTOF–MS) showed that the amount of asparaptine (**1**) (0.479%) was in the crude extract. Nicotianamine and 2″-hydroxynicotianamine were not detected in this analysis. The contribution of asparaptine (**1**) to the total ACE inhibitory activity of asparagus accounted for 2.2%, indicating that asparaptine (**1**) is another potent inhibitor of ACE in asparagus in addition to previously characterized compounds such as nicotianamine derivatives. In this analysis, flavonoids such as rutin [quercetin 3-*O*-rhamnosyl-(1→6)-glucoside] were also detected. Flavonoid constituents also have an inhibitory activity against ACE; however, their inhibitory activity is much weaker than that of asparaptine (**1**).[17] The total flavonoid glycosides in the crude extracts seemed to have a partial role in the inhibitory activity.

The amount of asparaptine (**1**) in the green asparagus spears was 251 mg/kg fresh weight (Table 2). It has been reported that the amount of 2″-hydroxynicotianamine in green asparagus is 47.0–54.0 mg/kg fresh weight, and that of nicotianamine is less than or equal to one-tenth of that in green asparagus.[5] The amount of asparaptine (**1**) was approximately 5-fold higher than that of 2″-hydroxynicotianamine. The amount of asparaptine (**1**) tends to be higher in white asparagus than in green or purple asparagus as well as in asparagusic acid and its glucose ester (Table 2). Conversely, the amount of rutin was the lowest in the white asparagus. Rutin is a light-inducible flavonol glycoside,[18] and white asparagus is generally grown under sunlight-shielded conditions. These facts suggest that the biosyntheses of asparaptine (**1**) and the other S-metabolites are unaffected by sunlight irradiation.

Table 2. Quantification of Specialized Metabolites in Asparagus

compound	amount (mg/kg fresh weight)		
	green	purple	white
asparaptine (**1**)	251 ± 17	185 ± 26	298 ± 22
asparagusic acid	98.6 ± 11	84.6 ± 8.4	106 ± 13
asparagusic acid glucose ester	289 ± 23	301 ± 24	324 ± 20
rutin	482 ± 48	460 ± 48	11 ± 1.3

The biosynthetic pathway of asparaptine (**1**) remains to be elucidated, but its structure implies the occurrence of a specific arginine *N*-acyltransferase, which catalyzes the conjugation of asparagusic acid with arginine. Comparative analysis using non- and accumulating organs in the integrated analysis of transcriptomics and metabolomics should enable streamlining the identification of the gene. The biosynthetic pathway of asparagusic acid is under investigation, and stable isotope labeling experiments suggest that asparagusic acid and its analogues are derived from valine through isobutyric acid, methacrylic acid, 2-methyl-3-mercaptopropionic acid, and *S*-(2-carboxy-*n*-propyl)cysteine.[19] Along with the biosynthetic pathway and enzymes of asparaptine (**1**), the investigation of the biological role of asparaptine (**1**) in asparagus is also intriguing with regard to the general function of S-metabolites in monocotyledons.

In summary, solid evidence of the existence of a new S-containing inhibitor, asparaptine (**1**), of ACE has been provided in asparagus using S-omics, a targeted metabolomic approach. So far, the number of compounds from plants beneficial to humans discovered by the top-down metabolomics approach is limited.[20] To our knowledge, this study is the first example showing that S-omics could be used for efficient screening of unknown S-metabolites in plants. By developing databases storing metabolic information such as compound name, structure, elemental composition, and MS/MS spectrum details, it may be understood whether target metabolites are known or unknown. Given that newly approved drugs are developed on the basis of the structures of natural products containing heteroatoms (N, O, S, and halogens),[21] this type of approach could be used for the screening of seed compounds containing heteroatoms for drug development and for functional food health benefit. It is recognized that S-omics is limited to redundant S-metabolites showing ^{34}S isotopic ions in

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the MS spectrum. However, profiling results of the redundant S-metabolites could be good markers for isolation of target metabolites. S-Omics and the discovery of asparaptine (**1**) will present opportunities to find new S-metabolites with biological activities potentially beneficial to humans.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotation was measured using a JASCO P-1020 polarimeter. NMR spectra were recorded using a Bruker 600 MHz spectrometer with a DCH CryoProbe (Bruker BioSpin GmbH, Rheinstetten, Germany). MS and MS/MS data were recorded using a Bruker solariX 7.0 T FTICRMS instrument (Bruker Daltonik GmbH, Bremen, Germany). Purification was performed on a preparative LC−PDA−MS system comprising two LC-20AP binary gradient pumps, an LC-20AD makeup pump, an SPD-M20A photodiode array detector, an SIL-10AP autosampler, a CTO-20A column oven, an LC-MS-2020 single quadrupole mass spectrometer, an FRC-10A fraction collector, and a CBM-20A module (Shimadzu, Kyoto, Japan). *N*-Succinyl-L-proline was purchased from Sigma-Aldrich Co., LLC (Saint Louis, MO, USA), and captopril, nicotianamine, D-arginine, L-arginine, and L-aspartic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Asparagusic acid was purchased from Shanghai Haoyuan Chemexpress Co., Ltd. (Shanghai, China). Isolated asparagusic acid glucose ester was used in this study.

MS and MS/MS spectra were recorded using Hystar 3.0 software (Bruker Daltonik GmbH). Data were processed using DataAnalysis 4.0 (Bruker Daltonik GmbH). The raw files were converted to netCDF. Peak picking of S-ions was performed using the theoretical mass difference (1.995 ± 0.001 Da) between ^{32}S-monoisotopic ions and their ^{34}S-substituted counterparts and using the natural abundance of ^{34}S (4.21 ± 5%) with the appropriate Web-based analytical tool for heteroatom-containing metabolites, which will be publicly available after reaching the final step. S-Ions were extracted under the condition that the retention time was 1−20 min. Peak alignment was conducted using Progenesis CoMet (Nonlinear Dynamics, Durham, NC, USA). Signal intensity values of all ions were divided by the signal intensity value of the internal standard lidocaine for normalization. To obtain S-ions with redundant signal intensity, ions with an intensity less than 1.0 were cut out. The software MeV 4.8 (http://www.tm4.org/mev.html) was used for hierarchical cluster analysis. Pearson's correlation analysis was used in this study. The elemental composition was calculated using SmartFormula (Bruker Daltonik GmbH) with the following limiting conditions: <1 mDa; $C_{0-50}H_{0-100}N_{0-5}O_{0-50}S_{0-5}$; charge, 1.

Plant Material. Commercially available spears of *Asparagus officinalis* produced in Japan (Hokkaido for both S-omics using LC−FTICRMS and quantification analysis using LC−QTOF−MS, Tochigi for isolation) were used in this study. Voucher specimens [green asparagus (Hokkaido), APGDo-FD; purple (Hokkaido), APPDo-FD; white (Hokkaido), APWDo-FD; and green (Tochigi), APGTo-FD] were deposited at RIKEN Freeze-dried Collection (RIKEN CSRS). Vegetables were purchased at U-Takaraya (Arakawa-ku, Tokyo, Japan). *Arabidopsis thaliana* and *Oryza sativa* L. were grown in a plant growth room.[22,23] All samples were immediately frozen, lyophilized, and ground and then stored at room temperature with silica gel until use.

Extraction and Isolation. Extraction of metabolites for LC−FTICRMS analysis was performed as previously described.[10] The column was exchanged for Xselect CSH Phenyl-Hexyl (3.5 μm, 2.1 mm × 150 mm, Waters, Milford, MA, USA).

Fresh asparagus spears (970.7 g) were cut into 2−3 mm pieces with a ceramic knife. After flash-freezing in liquid nitrogen, the pieces were freeze-dried and crushed. The lyophilized asparagus powder (57.5 g) was extracted three times with 80% methanol (3 L × 3) overnight at room temperature. The solvent was evaporated until a small volume of water solution was left (approximately 400 mL). The solution was sequentially extracted with *n*-hexane (400 mL × 3) and chloroform (400 mL) to remove lipid and chlorophyll. After the liquid−liquid extraction and evaporation, the water phase (approximately 50 mL) was subjected to open-column chromatography [column diameter 7.3 cm, length 15 cm, Cosmosil 75C_{18}-OPN materials, (Nacalai Tesque, Kyoto, Japan)] and eluted with MeOH−H_2O (0:100 → 100:0 v/v) to afford 13 fractions (Frs. 1 and 2, 0% MeOH; Frs. 3 and 4, 5% MeOH; Frs. 5 and 6, 10% MeOH; Frs. 7 and 8, 20% MeOH; Frs. 9 and 10, 40% MeOH; Frs. 11 and 12, 60% MeOH; Fr. 13, 100% MeOH; 500 mL solvent for each elution). Next, the fractions were analyzed with a Shimadzu LC-MS-2020 system. The protonated molecular ion of asparaptine (**1**) was observed at *m*/*z* 307 $[M + H]^+$ and was mainly found in fraction 6 (10% MeOH). After evaporation, fraction 6 (ca. 12 mL) was purified using a mass-directed fractionation system, the preparative LC−PDA−MS system (Shimadzu), to yield 61 mg of asparaptine (**1**). The purification was performed under the following conditions: LC-20AP pump A and B; Unison UK-C_{18} column [150 × 10 mm i.d.; 3 μm (Imtakt, Kyoto, Japan)]; column oven temperature of 40 °C, linear gradient solvents at a flow of 3 mL/min [solvent A: water (0.1% HCOOH); B: acetonitrile (0.1% HCOOH) 5% B (0−2 min), 5−8% B (2−3 min), 8−10% B (3−21 min), 10−100% B (21−22 min), 100% B (22−27 min), 100−5% B (27−28 min), 5% B (28−35 min)]; a makeup flow (LC-20AD pump C, solvent B, 0.2 mL/min) was used to split the LC eluent into an LC-MS-2020 mass spectrometer at a ratio of 1:150; electrospray ionization mass spectra were recorded through a range of *m*/*z* 100−1000 in the positive-ionization mode with a probe voltage of 4.5 kV, nebulizing gas flow of 1.5 L/min, "DL" temperature of 250 °C, heat block temperature of 200 °C, and drying gas flow of 20.0 L/min.

Asparaptine (1): white powder; $[\alpha]^{20}_D$ +6.5 (*c* 0.03, H_2O); ^{1}H NMR (600 MHz, in D_2O containing 0.01% DDS-d_6, 25 °C) δ 4.21 (1H, dd, *J* = 8.2, 5.0 Hz, H-8), 3.46 (1H, m, H-5a), 3.44 (1H, m, H-4), 3.42 (1H, m, H-3a), 3.34 (1H, m, H-3b), 3.28 (1H, m, H-5b), 3.21 (2H, t, *J* = 6.9 Hz, H-11), 1.86 (1H, m, H-9a), 1.72 (1H, m, H-9b), 1.61 (2H, m, H-10); ^{13}C NMR (150 MHz, in D_2O containing 0.01% DDS-d_6, 25 °C) δ 181.2 (C, C-14), 176.7 (C, C-6), 159.5 (C, C-13), 57.6 (CH, C-8), 54.0 (CH, C-4), 45.4 (CH_2, C-5), 44.6 (CH_2, C-3), 43.3 (CH_2, C-11), 31.5 (CH_2, C-9), 27.2 (CH_2, C-10); FTICRMS *m*/*z* 307.08931 ($[M + H]^+$, calcd for $C_{10}H_{19}N_4O_3S_2$, 307.08930).

Hydrolysis of Asparaptine (1). Asparaptine (**1**) (1.9 mg) was suspended in 6.0 N HCl (2.0 mL) and heated at 85 °C for 8 h. The acid hydrolysate was dried under N_2 and dissolved in H_2O−MeOH−HCOOH (30:70:0.02). The sample was then analyzed by HPLC under the following conditions: LC-20AD pump A and B; Astec CHIROBIOTIC T column (150 × 2.1 mm i.d., 1024AST, Sigma-Aldrich); column oven temperature of 25 °C; solvent H_2O−MeOH−HCOOH (30:70:0.02) at a flow rate of 0.2 mL/min; UV detection at a wavelength of 200 nm. D- and L-Arginine showed retention times at 11.1 and 8.1 min, respectively. L-Arginine in the hydrolysate was identified by comparing its retention time with that of an authentic sample.

Quantification of S-Metabolites in Asparagus Using LC−QTOF−MS. The fresh samples were extracted with 5 μL of 80% MeOH containing 2.5 μM lidocaine and 10-camphor sulfonic acid per mg fresh weight using a mixer mill with zirconia beads for 7 min at 18 Hz and 4 °C. After centrifugation for 10 min, the supernatant was filtered using an HLB μElution plate (Waters). Extract solutions (1 μL) were analyzed using LC−QTOF−MS (LC, Waters Acquity UPLC system; MS, Waters Xevo G2 Q-TOF). Analytical conditions were as follows: LC column, Acquity bridged ethyl hybrid C_{18} (1.7 μm, 2.1 mm × 100 mm, Waters); solvent system, solvent A (water including 0.1% formic acid) and solvent B (acetonitrile including 0.1% formic acid); gradient program, 99.5% A/0.5% B at 0 min, 99.5% A/0.5% B at 0.1 min, 20% A/80% B at 10 min, 0.5% A/99.5% B at 10.1 min, 0.5% A/99.5% B at 12.0 min, 99.5% A/0.5% B at 12.1 min and 99.5% A/0.5% B at 15.0 min; flow rate, 0.3 mL/min at 0 min, 0.3 mL/min at 10 min, 0.4 mL/min at 10.1 min, 0.4 mL/min at 14.4 min, and 0.3 mL/min at 14.5 min; column temperature, 40 °C; MS detection: capillary voltage, +3.0 keV, cone voltage, 25.0 V, source temperature, 120 °C, desolvation temperature, 450 °C, cone gas flow, 50 L/h; desolvation gas flow, 800 L/h; collision energy, 6 V; mass range, *m*/*z* 100−1500; scan duration, 0.1 s; interscan delay, 0.014 s; data

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acquisition, centroid mode; polarity, positive/negative; lockspray (leucine enkephalin): scan duration, 1.0 s; interscan delay, 0.1 s. MS/MS data were acquired in the ramp mode under the following analytical conditions: mass range, m/z 50−1500; collision energy, 10 to 50 V; scan duration, 0.1 s; interscan delay, 0.014 s; data acquisition, centroid mode; polarity, positive for asparaptine (**1**) and negative for asparagusic acid and asparagusic acid glucose ester. The solvents of asparaptine (**1**), asparagusic acid, and asparagusic acid glucose ester (<100 μM) were analyzed to produce a standard curve. These metabolites were then quantified using this standard curve. Differences and standard deviations were calculated from the results of three replicates.

ACE in Vitro Assay. The assays of the ACE inhibitory activity on concentrations of the compounds (<1 mM) and crude extracts (<1000 μg/mL) were conducted using an ACE Kit-WST (Dojindo Molecular Technologies, Kumamoto, Japan). The fresh samples were extracted with 5 μL of 80% MeOH per mg fresh weight using a mixer mill with zirconia beads for 7 min at 18 Hz and 4 °C. After centrifugation for 10 min, the supernatant was filtered using the HLB μElution plate. After concentration, crude extracts were dissolved with water, and the extracts were used for the assay. The IC_{50} values were determined according to the manufacturer's instructions. However, in the case where 50% inhibition was not reached at the high concentrations, the IC_{50} value was considered as N.D. (not determined). Differences and standard deviations were calculated from the results of three replicates.

Calculation of Contribution Ratio of Asparaptine (1) to ACE Inhibitory Activity of Asparagus Crude Extract. The contribution ratio was calculated as follows:

$$\text{contribution ratio (\%)} = IC_{50}\text{ value of the crude extract (}\mu\text{g/mL)} \times \text{the amount of asparaptine (}\mathbf{1}\text{) in the crude extract (\%)} / IC_{50}\text{ value of the asparaptine (}\mathbf{1}\text{) (}\mu\text{g/mL)}$$

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The ^{1}H, ^{13}C, and 2D NMR spectra of compound **1** are available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00092.

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Notes

The authors declare no competing financial interest.

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