Metabolism of airborne methyl salicylate in adjacent plants

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Abstract

Salicylic acid (SA) and methyl salicylate (MeSA) are synthesized in many plants and are crucial components that establish their disease responses. The metabolism of airborne MeSA to SA has been previously reported. In this report, it was found that SA glucose ester (SAGE), ether (SAG), and salicyloyl-L-aspartic acid (SA-Asp) are metabolites of airborne MeSA. Furthermore, it was found that airborne MeSA was able to increase the endogenous amount of rosmarinic acid in *Perilla frutescens*, which is known as one of the functional components that contributes to the maintenance of human health.

Keywords; airborne methyl salicylate; salicylic acid; *Arabidopsis thaliana*; *Perilla frutescens*; volatile organic compounds

Plants have unique and complex chemical systems to act against various stresses, such as biotic and abiotic stresses [1]. Methyl salicylate (MeSA) is a volatile organic compound that plays critical roles in plants to establish defense systems against biotrophic attacks, such as viral attacks [2]. MeSA is synthesized in plants from salicylic acid (SA), and the biosynthesis of SA starts from chorismic acid and is followed by a series of chemical reactions through two distinct pathways: the isochorismate (IC) pathway and the phenylalanine ammonia-lyase (PAL) pathway (Figure 1) [3]. In the IC pathway, SA is synthesized from chorismic acid via isochorismic acid [4]. However, recently, Rekhter et al. [5] reported an additional metabolite, isochorismate-9-glutamate in cytosol that converts from isochorismate and further metabolizes into SA. In contrast, in the PAL pathway, prephenic acid is synthesized from chorismic acid and then converted into L-phenylalanine (Phe). Subsequently, Phe is further converted into cinnamic acid [6]. The cinnamic acid is then converted into benzoic acid, which is further converted to SA [7]. Several SA metabolites are known, such as MeSA, which is produced via reactions catalyzed by carboxyl methyltransferases [8]. As with other kinds of metabolites, SA glucose ether (SAG), SA glucose ester (SAGE), and salicyloyl-L-aspartic acid (SA-Asp) have been reported [9]. SAG is the major conjugate of SA and glucose, which is catalyzed by two UDP-glucosyltransferases, UGT74F1 and UGT74F2, while its minor conjugate is salicylic glucose ester (SGE), which is catalyzed by UGT74F2 in *Arabidopsis thaliana* upon pathogen attack [9]. SA-Asp is the only SA-amino acid conjugate reported in various plants, such as *Vitis* (grape) species, *Phaseolus vulgaris* and *A. thaliana* [9], although several conjugated forms of jasmonic acid with amino acids have been reported [10]. The GH3.5 protein in *A. thaliana*, a multifunctional acetyl-amido synthetase, has been found to show adenylating activity on SA *in vitro* [11]. It has been found that SA-Asp induced *PR* gene expression in *A. thaliana* and enhanced disease resistance against *Pseudomonas syringae* [12].

As mentioned above, MeSA is a pivotal component in preparing biotrophic attacks. Shulaev et al. [13] reported that MeSA was the major volatile compound produced by tobacco plants inoculated with the tobacco mosaic virus. Another study showed that the production of MeSA significantly increases in *P. syringae* inoculated onto *A. thaliana* leaves and that it activated systematic acquired resistance (SAR) [14]. In addition to being a volatile compound, it has been reported that MeSA is a mobile component through the phloem to activate SAR in undamaged leaves [4, 8]. Recently, Salamanca et al. [15] reported that MeSA has the ability to attract beneficial arthropods, natural enemies of herbivores. Therefore, it is generally accepted that MeSA activates defense responses in healthy tissues of infected plants and in neighboring plants as an airborne signal [13].

Thus far, most studies have focused on MeSA synthesis and emission triggered by biotrophic damage. However, there is a very limited amount of information on the metabolic fate of airborne MeSA. To the best of our knowledge, there have been no reports other than the metabolic pathway from MeSA to SA [13]. Here, we report the metabolism of airborne MeSA into SAGE, SAG, and SA-Asp using *A. thaliana*. In addition, we proposed that airborne MeSA might be used in agriculture.

Materials and Methods

General

NMR spectra were recorded using a JNM-EX 270 FT-NMR spectrometer (JEOL, Tokyo, Japan, ^{1}H NMR: 270 MHz, ^{13}C NMR: 67.5 MHz). FDMS and FIMS analyses were performed on a JMS-T100GCV (JEOL, Tokyo, Japan), and CIMS was performed on a JMS-SX102A (JEOL, Tokyo, Japan) instrument. [$^{2}H_{1}$-3, $^{2}H_{1}$-4, $^{2}H_{1}$-5, $^{2}H_{1}$-6]-SA (SA-d_4), SAG and SA were purchased from Medical Isotope Inc. (Pelham, NH, USA), Toronto Research Chemicals (Toronto, ON, Canada), and Kanto Chemical Co., Inc. (Tokyo, Japan) respectively. MeSA, acetyl salicylic acid and diethyl-L-aspartate hydrochloride were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Preparation of filter papers containing MeSA and MeSA-d_4

An aliquot (0.1 mL) of a solution containing MeSA (1 mg/0.1 mL Et_2O) was dropped onto a filter paper (ϕ 10 mm), and the paper was left for 10 min at room temperature to allow the organic solvent to evaporate. An aliquot (0.1 mL) of the solution containing [$^2H_1$-3, $^2H_1$-4, $^2H_1$-5,$^2H_1$-6]-SA (SA-d_4) (1 mg/0.1 mL MeOH) was dropped onto a filter paper (ϕ 10 mm), and an excess amount of CH_2N_2 in Et_2O was added to give MeSA-d_4. The conversion yield of MeSA-d_4 from SA-d_4 was evaluated to be 92%, which was estimated by GC-MS analysis using MeSA as a standard. GC-MS analysis was carried out according to the method given in supplemental material. The paper was left for 10 min at room temperature to allow the organic solvent to evaporate for use in the treatment of airborne [$^2H_1$-3, $^2H_1$-4, $^2H_1$-5,$^2H_1$-6]-MeSA (MeSA-d_4).

Determination of metabolites of airborne MeSA and MeSA-d_4

A. thaliana plants were grown at 23 °C with a 16 h light and 8 h dark photoperiod for thirty days. Six plants were placed in a semiclosed chamber (Triple L, 40.0 cm x 25.6 cm x 28.0 cm, Kotobuki Co., Ltd. Tenri, Japan) together with three filter papers containing MeSA (1 mg/filter paper) or MeSA-d_4 (1 mg/filter paper) for 24 h. A solution of Et_2O was used as a control treatment instead of MeSA or MeSA-d_4. The upper parts of the plants were harvested and extracted with EtOH. To determine the endogenous amounts of the metabolized compounds when using airborne MeSA, an aliquot solution containing [$^2H_1$-3, $^2H_1$-4, $^2H_1$-5,$^2H_1$-6]-SA (25 ng) and [$^2H_1$-3, $^2H_1$-4, $^2H_1$-5, $^2H_1$-6]-SAG (200 ng) was added. UPLC MS/MS analysis was performed with MRM in negative mode according to a previously reported method [16].

Synthesis of salicyloyl-L-aspartic acid (SA-Asp)

Synthesis of acetyl salicyloyl-L-diethyl aspartate (ASA-Asp(OEt)$_2$)

To synthesize acetyl salicyloyl-L-diethyl aspartate (ASA-Asp(OEt)$_2$), a reaction mixture

of acetyl salicylic acid (185.2 mg, 1.03 mmol), diethyl-L-aspartate hydrochloride (309.2 mg, 1.37 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (206.3 mg, 1.08 mmol) and 1-hydrochloride-1*H*-benzotriazole (140.1 mg, 1.04 mmol) in 10 mL of *N*,*N*-dimethylformamide was stirred overnight at room temperature. To the stirred mixture, EtOAc (50 mL) was added, and the mixture was washed with 90 mL (30 mL × 3) of 0.1 M HCl and 30 mL of saturated $NaHCO_3$, successively. After drying over with anhydrous Na_2SO_4, the EtOAc soluble layer was concentrated under reduced pressure. The resulting oil was purified by silica gel column chromatography using EtOAc:hexane, 7:13 (v/v) to yield ASA-Asp$(OEt)_2$ (153.6 mg, 42.50%). ^{1}H NMR (270 MHz, $CDCl_3$, Figure 2S in supplemental material) δ 7.96 (dd, *J* = 7.7, 1.8 Hz, 1H), 7.74 (br d, *J* = 7.3 Hz, 1H), 7.51-7.42 (m, 1H), 7.30 (t, *J* = 7.5 Hz, 1H), 7.12 (dd, *J* = 8.1, 1.2 Hz, 1H), 5.01-4.93 (m, 1H), 4.29-4.05 (m, 4H), 3.14-2.90 (m, 2H), 2.43-2.38 (m, 3H), 1.30-1.17 (m, 6H).

Synthesis of salicyloyl-L-aspartate (SA-Asp)

To a solution of ASA-Asp$(OEt)_2$ (141.3 mg, 0.40 mmol) in MeOH (360 µL), a solution of 0.5 M NaOH (4 mL) was added, and the reaction mixture stirred for 1 h. Volatile components of the reaction mixture were removed under reduced pressure. To the resulting oil, H_2O (10 mL) was added, and the mixture was washed with 30 mL of Et_2O. The aqueous solution was acidified using HCl (12 N) by adding dropwise at 0 °C, and the solution was extracted using EtOAc (30 mL × 3). The combined organic layers were dried over anhydrous Na_2SO_4, and the volatile components of the organic layer were removed to afford an oil. The resulting oil was purified using silica gel column chromatography (MeOH:$CHCl_3$; 1:1 (v/v) to afford SA-Asp (100 mg, 98.9%, $[\alpha]_D^{23}$ = +19.65, MeOH).

^{1}H NMR (270 MHz, CD_3OD, Figure 3S in supplemental material) δ 7.80 (dd, *J* = 8.2, 1.6 Hz, 1H), 7.41-7.26 (m, 1H), 6.92-6.88 (m, 1H), 6.88-6.81 (m, 1H), 4.99-4.90 (m, 1H), 2.94 (d, *J* = 5.6 Hz, 2H). ^{13}C NMR (67.5 MHz, CD_3OD, Figure 4S in supplemental

material) δ 174.62, 174.48, 170.31, 160.86, 135.20, 130.14, 120.58, 118.51, 117.61, 50.52, 37.09.

Detection of rosmarinic acid in P. frutescens using the UPLC MS/MS system

Perilla frutescens plants were grown at 23 °C with a 16 h light and 8 h dark photoperiod for thirty days. Six plants were put in a semiclosed chamber together with three filter papers containing MeSA (1 mg/filter paper) for 24 h. A solution of Et_2O was used as a control treatment instead of MeSA. The upper parts of the plants were harvested and extracted with EtOH. Before purification, a solution containing 100 ng of [$^2H_3$-12, $^2H_2$-11, $^2H_1$-10] jasmonic acid (JA-d_6) was added to the extract. The method for extraction and UPLC MS/MS conditions in positive mode were in accordance with a reported method [16]. The MS/MS parameters in MRM to detect JA-d_6 was done according to the reported method [16]. The MS/MS parameters in MRM to detect rosmarinic acid were as follows: parent ion: *m/z* 359.12, transition ion: *m/z* 160.77, cone voltage: 50.0 V and collision energy: 17.50 eV, in negative mode. Relative areas were calculated according to the following formula:

(Relative area) = (Area derived from rosmarinic acid) x 100 [ng] /(Area derived from JA-d_6) (fresh weight of the sample [g]) x 360 [g/mol]

Statistical analysis

All experiments depicted in the figures were conducted three times with similar tendencies. Statistical analyses were performed using Welch's t-test for comparison of two data sets.

Results

Metabolism of airborne MeSA into SA and its glucosides

SA plays critical plant defense roles against biotic and abiotic responses [6]. During pathogen infection, SA plays critical roles in SAR activation [17]. It has been reported that there is a higher accumulation of SA in tobacco plants inoculated with tobacco

mosaic virus (TMV) [18]. Moreover, Shulaev et al. [13] showed a higher accumulation of SA in tobacco plants incubated with gaseous MeSA. We planned to determine whether endogenous amounts of SAG and relative amount of SAGE together with SA increased with treatment using airborne MeSA. Plants were placed in a semiclosed chamber and treated with airborne MeSA as shown in Figure 1S. The plants were harvested, and the endogenous amounts of the target compound were evaluated according to a reported method [16]. The results are given in Figure 2, in which higher accumulations of SA, SAG and SAGE were observed in treated plants compared with nontreated plants. In the case of the accumulation of SA, a 36-fold higher accumulation of SA was found in the treated plants compared to the nontreated plants (Figure 2a). Since SA is active and toxic in some cases, it is generally accepted that the active compounds should be readily subjected to several modifications leading to its inactive forms, such as glucosylation, methylation and amino acid conjugation [14]. It has been reported that SAG is then actively transported to the vacuole from the cytosol until the plant requires its use in its active form of SA [19]. Consistent with this, our findings also showed a 28-fold higher accumulation of SAG than the control treatment in *A. thaliana* upon exposure to airborne MeSA (Figure 2b). Moreover, we detected relative accumulated amount of SAGE using SAG-d_4 as an internal standard. Based on the results we found that there was a 236-fold higher accumulation of SAGE compared to the control treatment (Figure 2c). However, there were two possibilities for this higher accumulation, which might be due to the upregulation of the SA biosynthetic pathways or exogenously inhaled MeSA.

Metabolism of airborne MeSA-d_4 into SA-d_4, glucose ester and ether and SA-Asp

To further understand whether airborne MeSA is metabolized in neighboring plants, MeSA-d_4 was used. The biological activities of MeSA-d_4 are thought to be the same as those of nonlabeled MeSA, and MeSA-d_4 are not converted into nonlabeled compounds [20]. Prior to the experiment, SA-Asp was synthesized according to the report of

Anabuki et al. [21]. Plants were placed in a semiclosed chamber and treated with airborne MeSA-d_4 as in the procedure used for application of airborne MeSA. The plants were harvested, and the endogenous target compounds were evaluated using the UPLC MS/MS system. The results are given in Figures 3-5, and we detected the peaks derived from SA, SA-d_4 (Figure 3), SAG, SAG-d_4, SAGE, SAGE-d_4 (Figure 4), SA-Asp, and SA-Asp-d_4 (Figure 5) from the leaves of *A. thaliana* treated with airborne MeSA-d_4 in the UPLC chromatogram with the same retention times as those of the authentic compounds. The conversion of ^{14}C-labeled MeSA to ^{14}C-labeled SA has been reported by Shulaev et al. [13], whose result coincided well with that of the conversion of MeSA-d_4 to SA-d_4 found in this study. We synthesized other kinds of SA-amino acid conjugates: SA-glutamic acid, SA-tryptophan, SA-isoleucine, SA-cysteine and SA-methionine, but no peaks corresponding to these conjugates were detected (data not shown).

Airborne MeSA stimulated the biosynthesis of rosmarinic acid

Treatment with airborne MeSA resulted in the accumulation of a vast amount of SA and SA metabolites derived from airborne MeSA itself compared to endogenous compounds (Figures 3-5). Thus, it was hypothesized that MeSA might stimulate other important components present the plants which might important to maintain human health. Rosmarinic acid is a polyphenolic phytochemical (Figure 6) that shows anti-inflammatory, anti-allergic and anti-oxidative activities [22]. It has been proven that rosmarinic acid is one of the main constituents of *P. frutescens* leaf extract [23]. In order to examine the above hypothesis, the endogenous amount of rosmarinic acid in *P. frutescens* plants treated with airborne MeSA was evaluated using the UPLC MS/MS system. Plants were exposed to airborne MeSA as previously described, and the leaves were harvested and extracted. Since there is no availability to get a stable isotope labelled rosmarinic acid, we used JA-d_6 as a putative internal standard to calculate relative endogenous amounts of rosmarinic acid. The results are given in Figure 6, which revealed a higher accumulation of rosmarinic acid in treated plants compared with that in nontreated plants. In addition to that, we evaluated the accumulation of SA

and its metabolites in *P. frutescens* treated with airborne MeSA. The results showed higher accumulation of SA, SAG and SAGE compare to the control treatment (Figure 5S in supplementary material). However, there was no peak corresponding to SA-Asp in *P. frutescens* treated with airborne MeSA.

Discussion

There were no other examples of the experimental method that we adopted, so it was unclear whether the plant actually elicited the wound response in our experimental system. Thus, the transcriptional levels of *PR1* and *PR5* in plants exposed to airborne MeSA were evaluated using a qRT-PCR system. The results are given in Figure 6S in the supplemental material, which indicated that a resistant response against biotrophs was established due to higher transcriptional levels of *PR1* and *PR5* in the treated plants compared with those in the nontreated plants. The results supported that the experimental method employed in this study was indicated to be as tolerable as any other study that discusses the metabolism and biological activity of airborne MeSA. With this experimental belief, it was first proven that airborne MeSA was metabolized not only to SA but also to SAG, SAGE, and SA-Asp. Since the biological activities of SAG and SAGE, have not been reported, it should be safe to say that these metabolites are inactive forms of SA. In addition, the promising usage of airborne MeSA in the agricultural industry has been suggested, such as to induce a higher accumulation of rosmarinic acid in *P. frutescens*, which is a well-known functional ingredient that contributes to the maintenance of human health [22-25].

Author Contributions

RM performed the experiments and wrote the paper. KO helped draft the manuscript. KT performed the experiments. HM designed the study and wrote the paper.

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UPLC MS/MS systems (Waters, USA) at the Research Faculty of Agriculture, Hokkaido University were used for this study.

Disclosure statement

No potential conflicts of interest are reported by the authors.

Supplemental materials

The supplemental materials for this paper are available at http://

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Figure legends

Figure 1. Biosynthesis pathway of salicylic acid.

Figure 2. Evaluation of SA, SAG and SAGE in *A. thaliana.*
Plants were exposed to airborne MeSA for 24 h. The upper parts of the plants were harvested and extracted with EtOH. Accumulated amounts of SA, SAG and SAGE were analyzed by UPLC-MS/MS. a) accumulated amount of SA. b) accumulated amount of SAG. c) relative accumulated amount of SAGE. Each value is represented as the mean ± SD of six independent replicates. Welch`s test, ** $p<0.01$.
x) The peak area was calculated corresponding to the internal standard of SAG-d_4.

Figure 3. Metabolism of airborne MeSA-d_4 into SA-d_4 in *A. thaliana*.
Plants were exposed to airborne MeSA-d_4 for 24 h and extracted with EtOH. Peaks were detected with a UPLC-MS/MS system in MRM negative mode. a) the peak was monitored by selecting *m/z* 140.8 as the pseudomolecular ion and *m/z* 96.6 as the transition ion for SA-d_4. b) the peak was monitored by selecting *m/z* 136.8 as the pseudomolecular ion and *m/z* 92.5 as the transition ion for endogenous SA.
x) The peak intensity is shown by converting the peak intensity value of 1.1×10^5 to 100%. y) Insets indicate the chemical structures of the target compounds.

Figure 4. Metabolism of airborne MeSA-d_4 into SAG-d_4 and SAGE-d_4 in *A. thaliana*.
Plants were exposed to airborne MeSA-d_4 for 24 h and extracted with EtOH. Peaks were detected with a UPLC-MS/MS system in MRM negative mode. a) the peaks were monitored by selecting *m/z* 302.9 as the pseudomolecular ion and *m/z* 140.8 as the transition ion for metabolized SAG-d_4 and SAGE-d_4. b) the peaks were monitored by selecting *m/z* 298.9 as the pseudomolecular ion and *m/z* 136.8 as the transition ion for endogenous SAG and SAGE.

x) The peak intensity is shown by converting the peak intensity value of 3.5×10^3 to 100%. y) Insets indicate the chemical structures of the target compounds.

Figure 5. Metabolism of airborne MeSA-d_4 into SA-Asp-d_4 in *A. thaliana*.
Plants were exposed to airborne MeSA-d_4 for 24 h and extracted with EtOH. Peaks were detected with a UPLC-MS/MS system in MRM negative mode. a) the peaks were monitored by selecting *m/z* 256.2 as the pseudomolecular ion and *m/z* 139.8 as the transition ion for metabolized SA-Asp-d_4. b) the peaks were monitored by selecting *m/z* 252.2 as the pseudomolecular ion and *m/z* 135.8 as the transition ion for endogenous SA-Asp.
x) The peak intensity is shown by converting the peak intensity value of 2.0×10^3 to 100%. y) Insets indicate the chemical structures of the target compounds.

Figure 6. Evaluation of rosmarinic acid in *P. frutescens* treated with airborne MeSA.
Plants were exposed to airborne MeSA for 24 h. The upper parts of the plants were harvested and extracted with EtOH. The accumulated amount of rosmarinic acid was analyzed with a UPLC-MS/MS in positive mode. The peak area was calculated corresponding to the internal standard of JA-d_6. Each value is represented as the mean ± SD of six independent replicates. Welch`s test, ** $p<0.01$.
x) Inset indicates the chemical structures of rosmarinic acid.

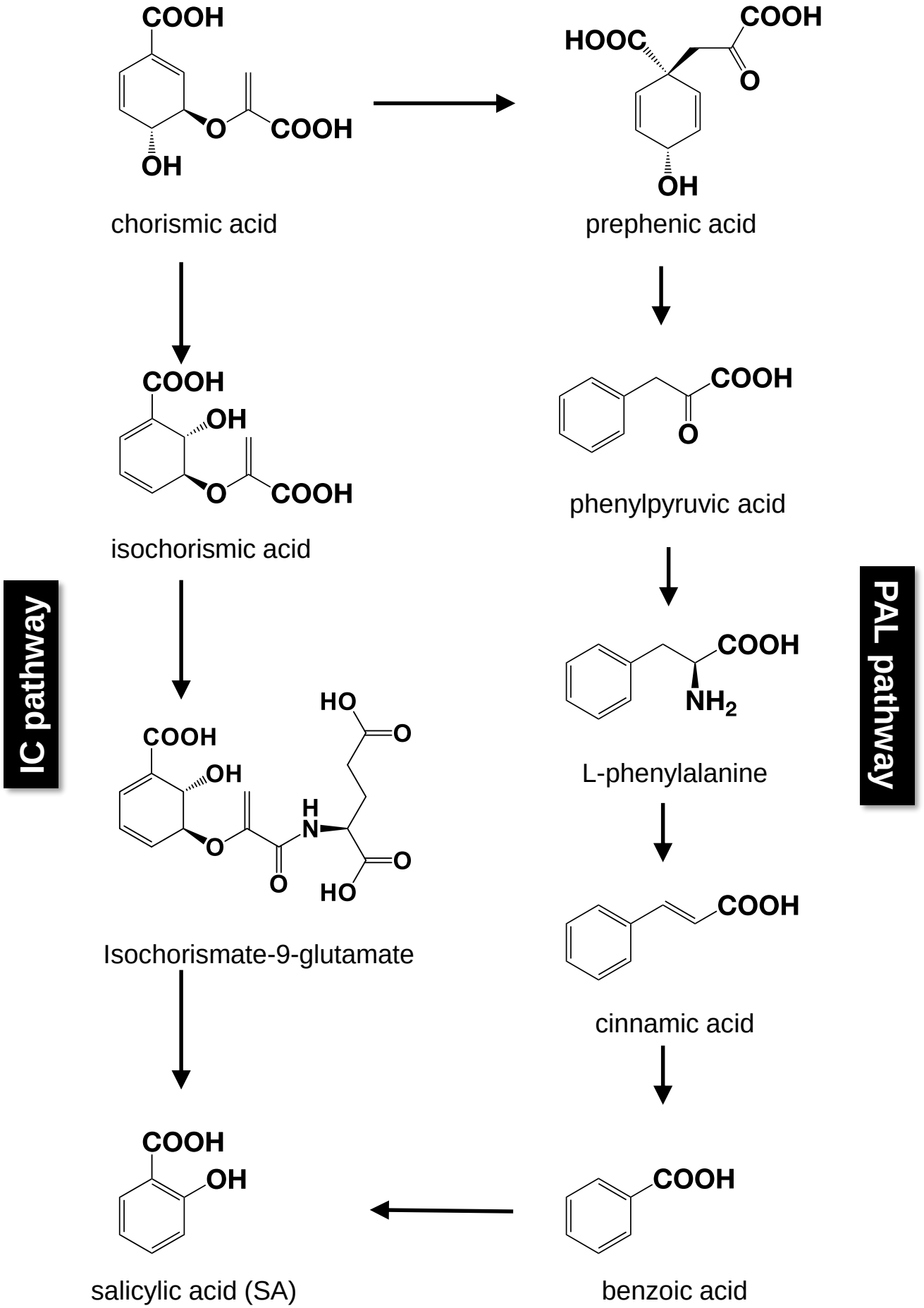


Figure 1. Biosynthesis pathway of SA.

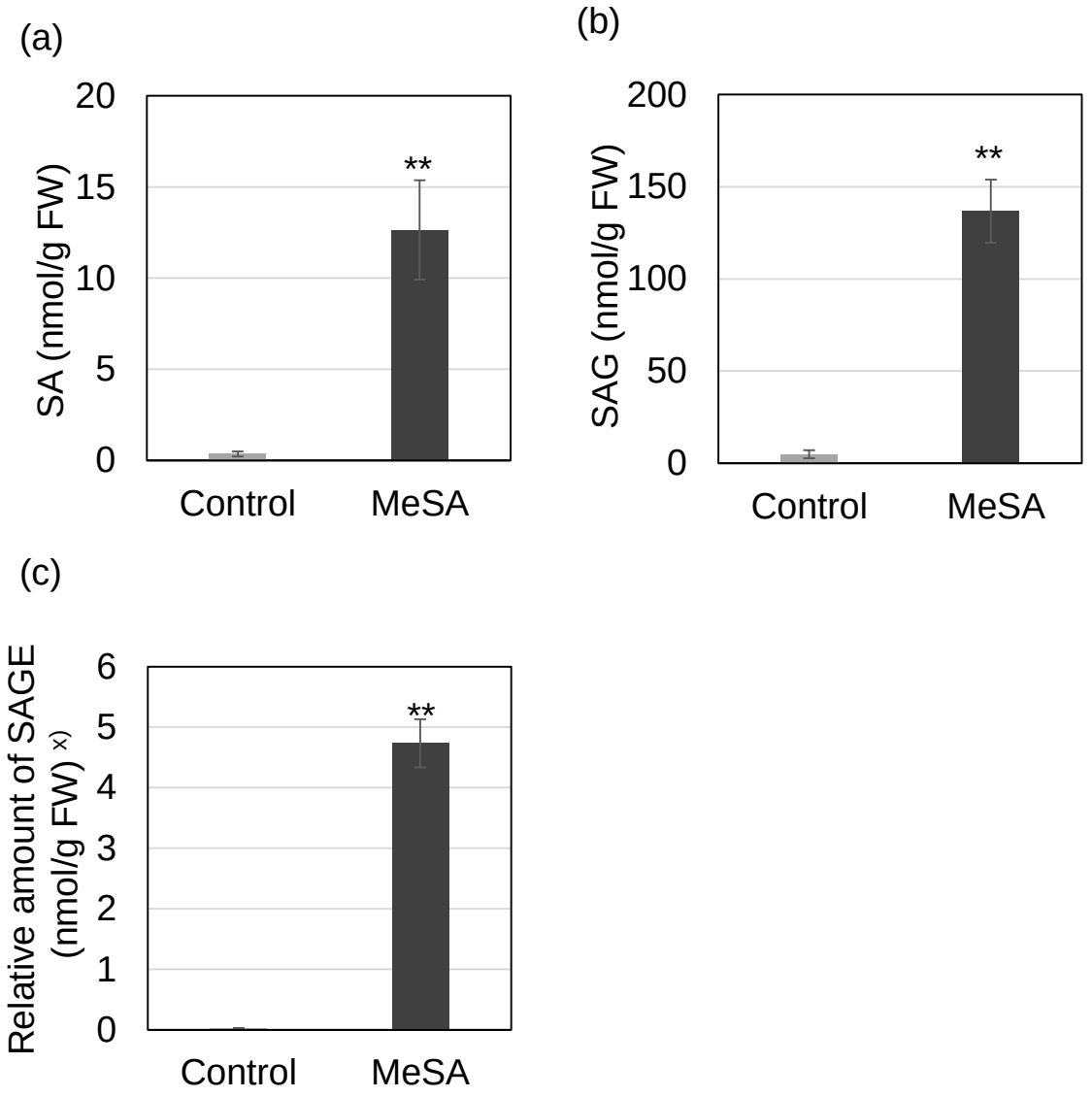


Figure 2. Evaluation of SA, SAG and SAGE in *A. thaliana.*

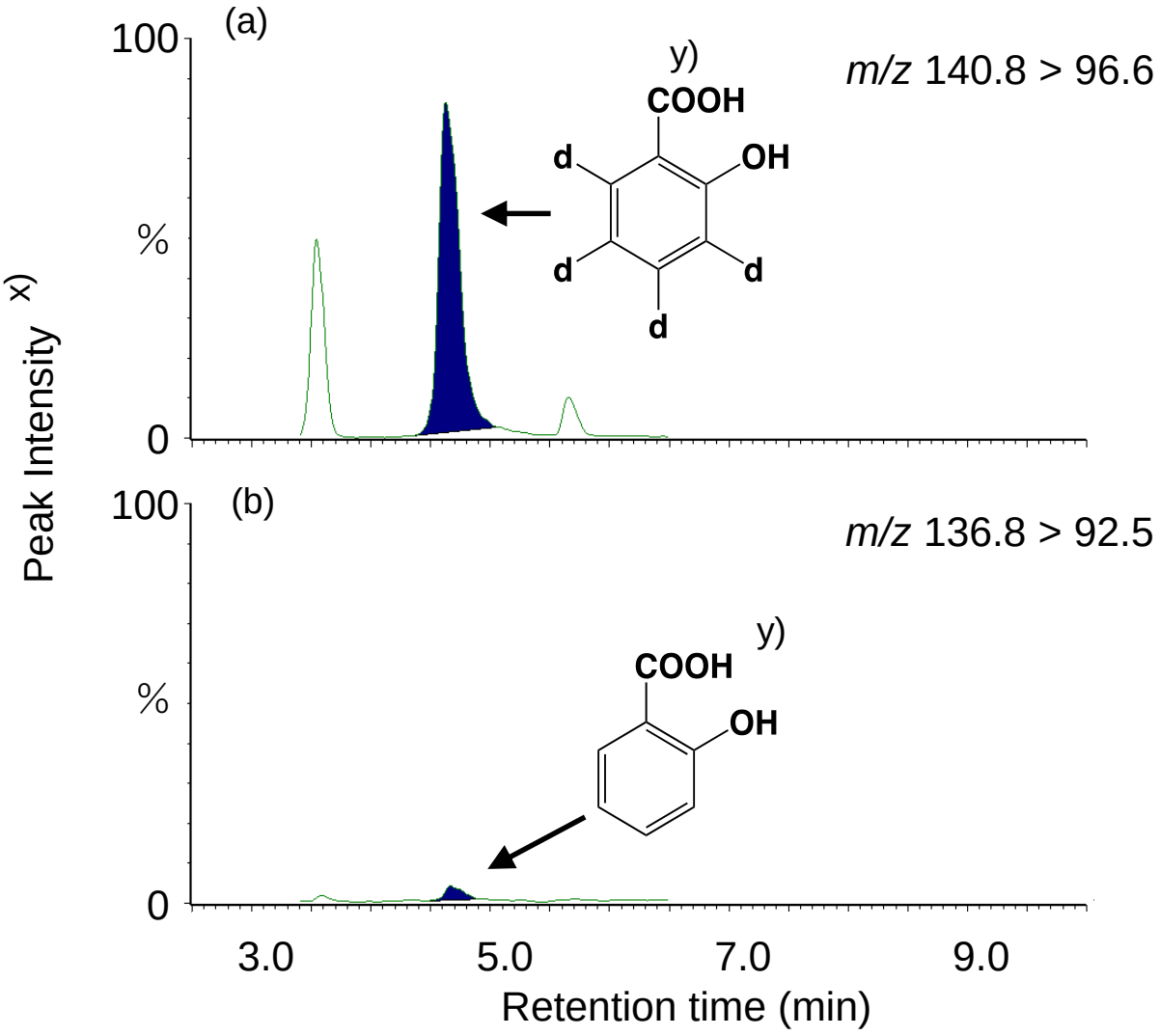


Figure 3. Metabolism of airborne MeSA-d_4 into SA-d_4. in *A. thaliana*.

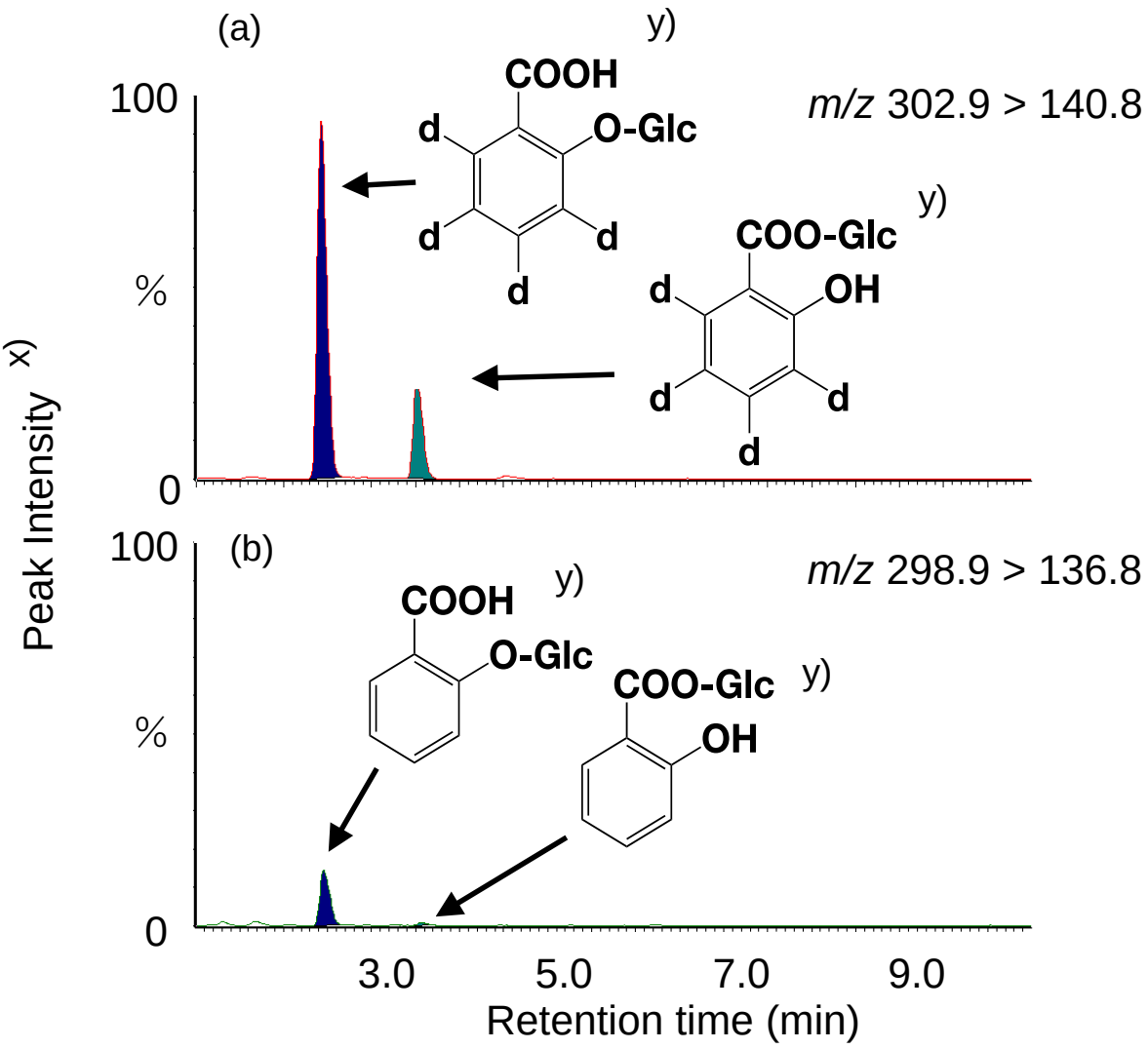


Figure. 4. Metabolism of airborne MeSA-d_4 into SAG-d_4 and SAGE-d_4 in *A. thaliana*.

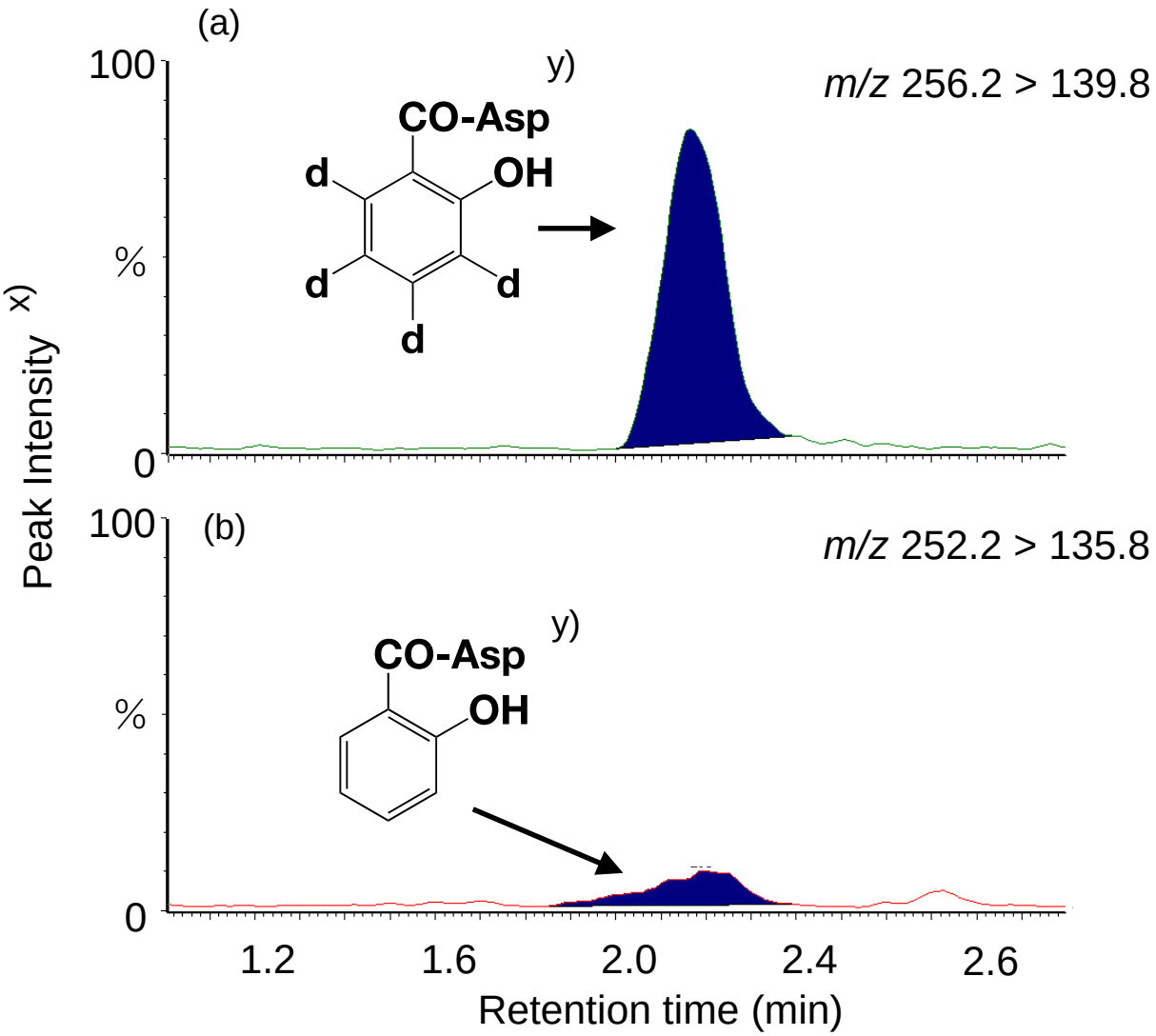


Figure 5. Metabolism of airborne MeSA-d_4 into SA-Asp-d_4 in *A. thaliana*.

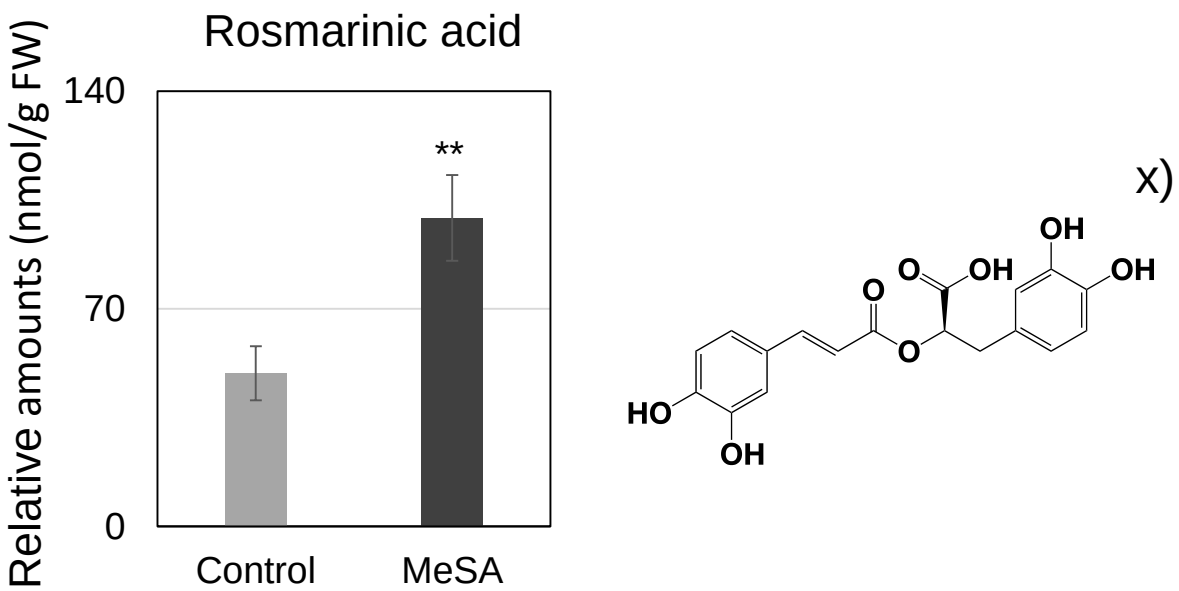
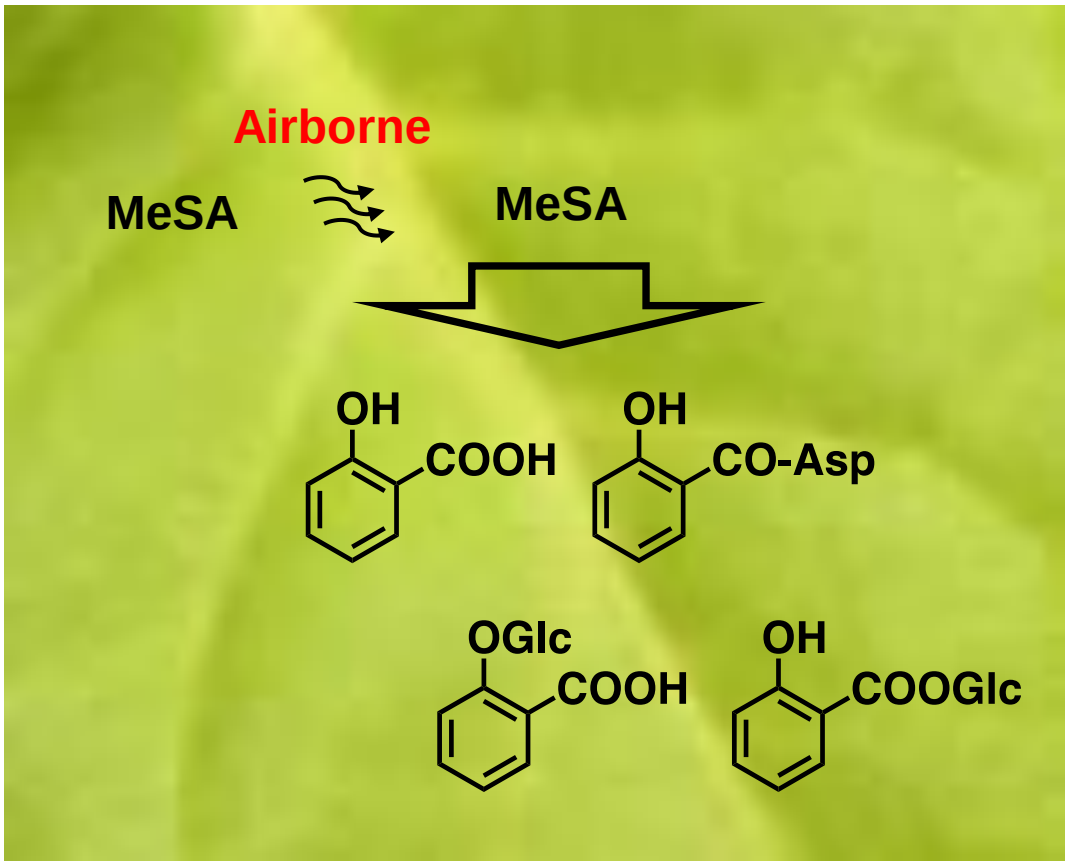


Figure 6. Evaluation of rosmarinic acid in *P. frutescens* treated with airborne MeSA.


Airborne
MeSA
MeSA
OH
COOH
OH
CO-Asp
OGlc
COOH
OH
COOGlc

Supplemental material

Metabolism of airborne methyl salicylate in adjacent plants.

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Figure 1S. Exposure of airborne MeSA or MeSA-d_4 to plants.

A. thaliana plants were grown at 23 ºC with a 16 h light and 8 h dark photo period for thirty days. Six plants were placed in a semiclosed chamber (Triple L, 40.0 cm x 25.6 cm x 28.0 cm, Kotobuki Co., Ltd. Tenri, Japan) together with three filter papers containing MeSA (1 mg/filter paper) or MeSA-d_4 (1 mg/filter paper) and kept for 24 h. The conversion yield of MeSA-d_4 from SA-d_4 was evaluated to be 92% according to the method shown in Experimental section in this supplemental material.

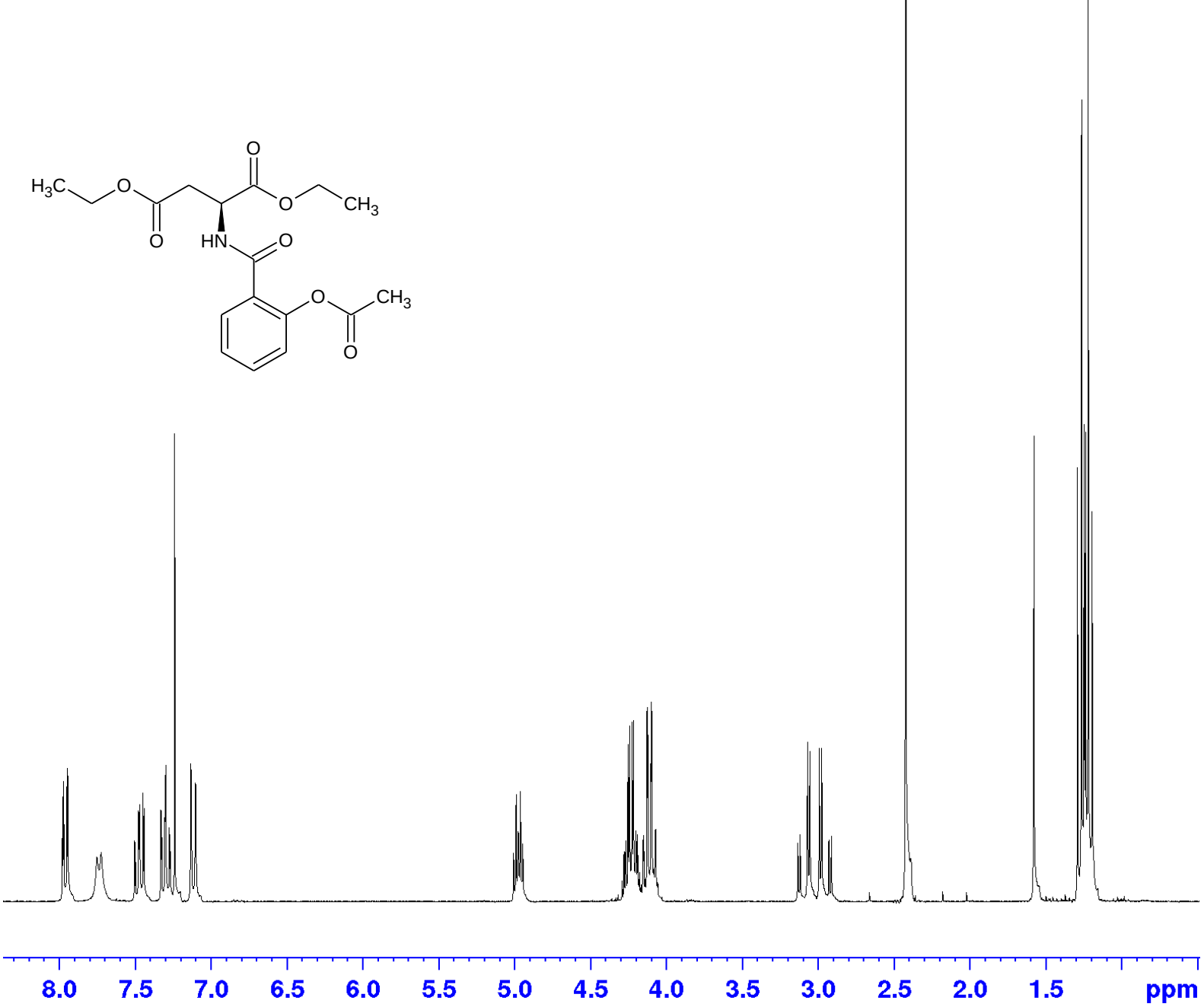

Figure 2S. ^{1}H NMR spectrum of ASA-Asp$(OEt)_2$ (270 MHz, $CDCl_3$).

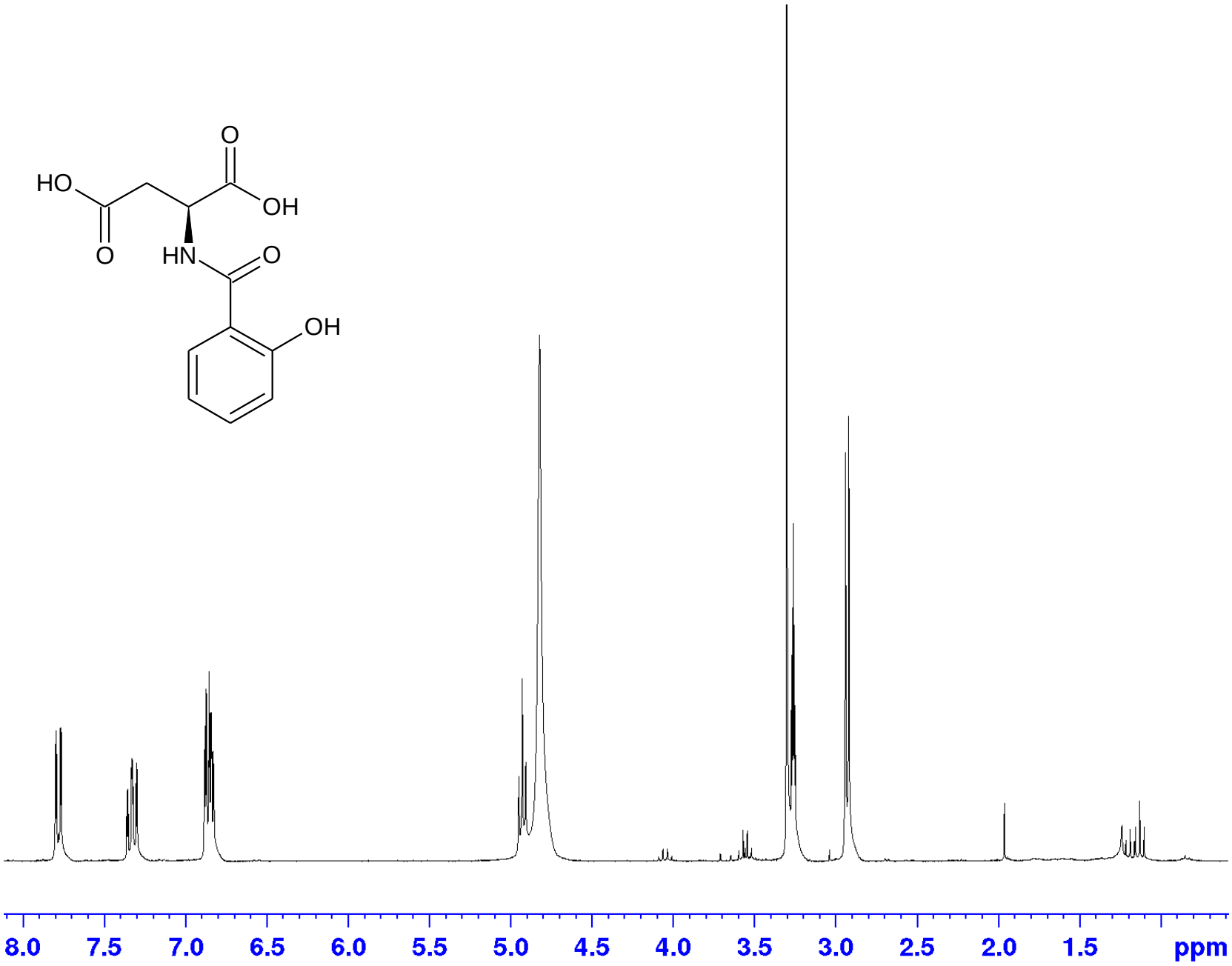

Figure 3S. ^{1}H NMR spectrum of SA-Asp (270 MHz, CD_3OD).

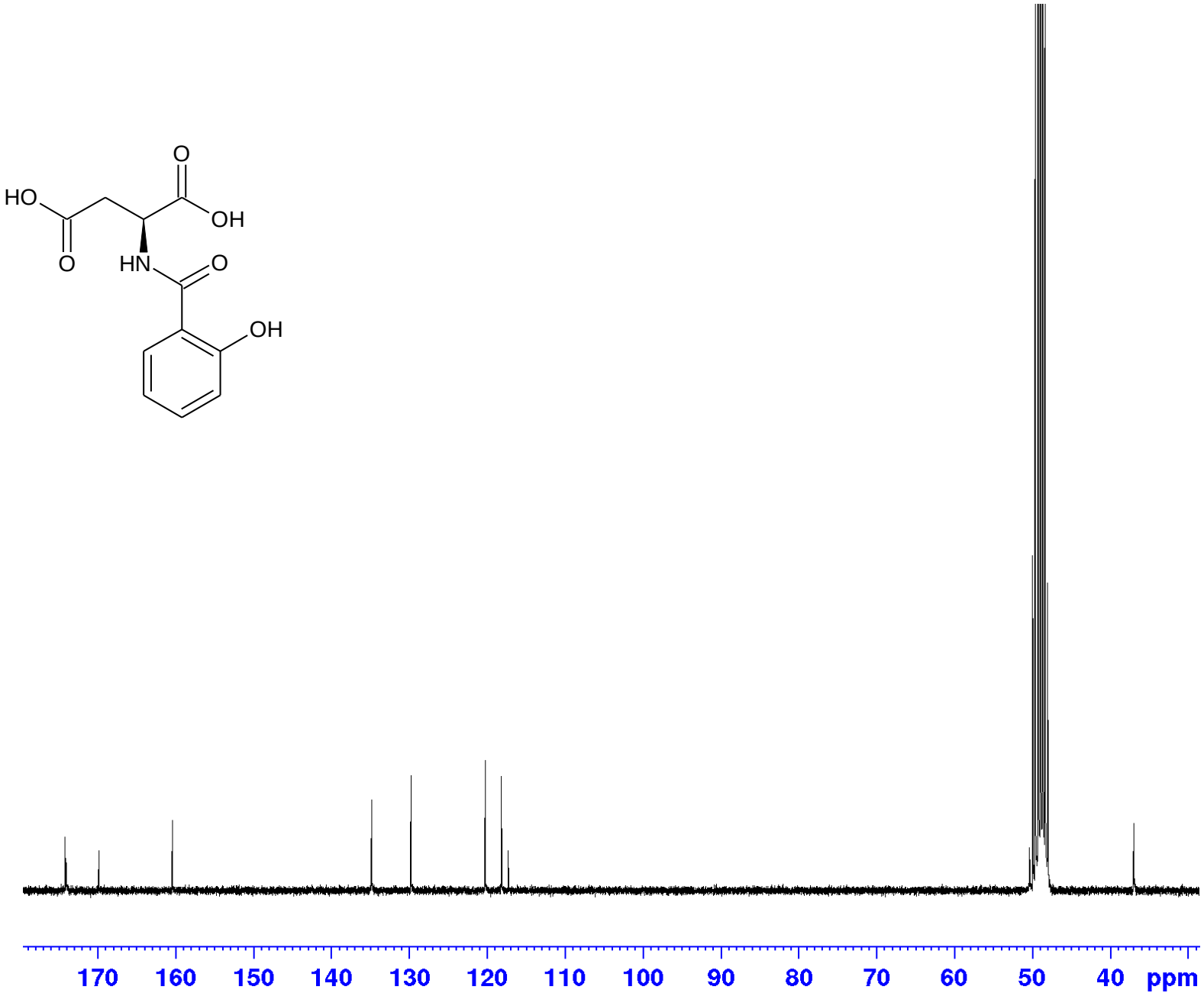

Figure 4S. ^{13}C NMR spectrum of SA-Asp (67.5 MHz, CD_3OD).

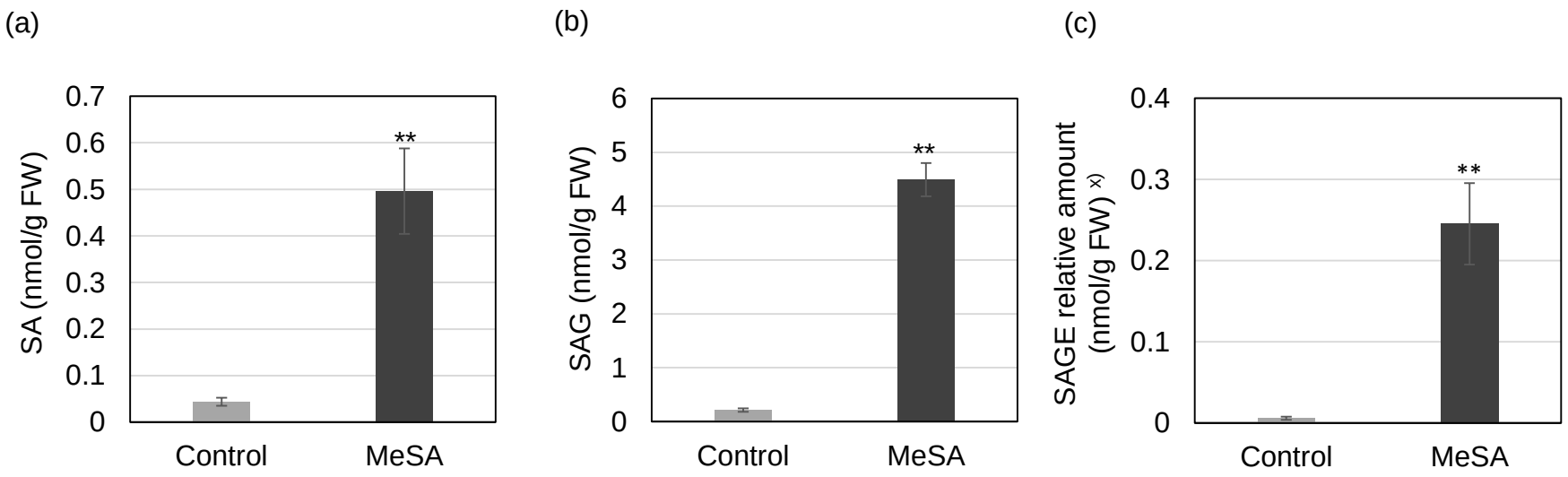

Figure 5S. Evaluation of SA, SAG and SAGE in *P. frutescens.*

Plants were exposed to airborne MeSA for 24 h. The upper parts of the plants were harvested and extracted with EtOH. Accumulated amounts of SA, SAG and SAGE were analyzed by UPLC-MS/MS. a) accumulated amount of SA. b) accumulated amount of SAG. c) relative amount of SAGE. Each value is represented by the mean ± SD of six independent replicates. Welch`s test, ** $p<0.01$

x) The peak area was calculated corresponding to the internal standard of SAG-d_4.

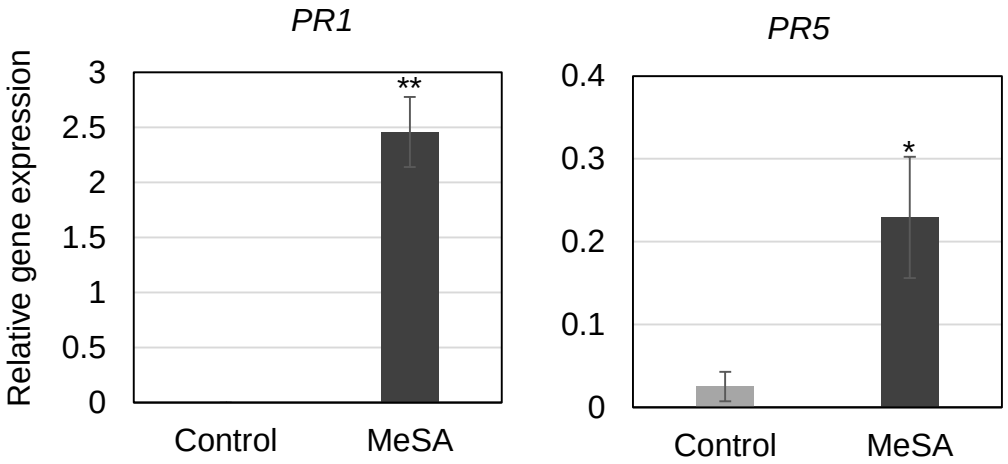

Figure 6S. Evaluation of transcriptional level of mRNA encoding *PR1* and *5.*

Plants were exposed to airborne MeSA for 24 h. Total RNA was extracted from frozen leaves (100 mg) of airborne MeSA treated and non-treated plants. mRNA was isolated using ISOSPIN Plant RNA extraction kit (Nippon gene, Japan) according to the manufacturer's protocol. The obtained RNA samples were subjected to reverse transcribed reaction using M-MLV reverse transcriptase (Invitrogen, USA). Quantitative real-time PCR was conducted using KOD SYBR qPCR Mix (Toyobo, Japan) as described in the manufacturer's protocol. Each reaction mixture contains 12.5 µL of KOD SYBR, 9.5 µL of MilliQ water, 1 µL of each primer (10 µM) and 1 µL of cDNA. qRT-PCR was performed in a Thermal Cycler Dice Real Time system (TAKARA TP800, Japan) at 95 ºC for 30 s followed by 40 cycles at 95 ºC for 5 s, 54 ºC for 30 s and 95 ºC for 15 s, 60 ºC for 30 s, 95 ºC for 15 s. Primers used in this study are listed in supplementary Table 1. An actin gene was used as a reference gene in this study. Each gene was calculated and expressed as fold regulation in comparison with the housekeeping gene actin. Each value is represented by the mean ± SD of six independent replicates. Welch`s test, * $p<0.05$, ** $p<0.01$,

Table 1S. Primer sets used for this study.

gene name			Annealing temperature [℃]
PR1 (AT2G14610)	forward:	GAGAAGGCTAACTACAACTACGC	54
	reverse:	CATTAGTATGGCTTCTCGTTCACA	
PR5 (AT1G75040)	forward:	CTGAATTCACTCTAGTAGGCGATG	54
	reverse:	TTGTTCTGATCCATGACCTTAAGC	
ACT2 (AT3G18780)	forward:	CTTGCACCAAGCAGCATGAA	54
	reverse:	CCGATCCAGACACTGTACTTCCTT	

Experimental

GC-MS analysis. GC-MS analysis was completed on a Varian CP-3800 gas chromatograph with a Varian 1200L quadrupole MS/MS in electron ionization mode. GC MS parameters for the detection as following. Injection temperature was 200 ºC, and a fused-silica capillary column (BetaDEX$_{120}$; 30 m × 0.25 mm i.d., 0.25 μm film thickness; GL Sciences) was used. The temperature program started at 80 ºC for 1 min and subsequently increased at 5 ºC /min to 220 ºC, which was maintained for 1 min. Helium was used as the carrier gas at a linear velocity of 1.2 mL/min, and all spectra were scanned within the range *m/z* 10-600.

Evaluation of conversion yield of MeSA-d_4 from SA-d_4. An aliquot (0.1 mL) of the solution containing [$^2H_1$-3, $^2H_1$-4, $^2H_1$-5,$^2H_1$-6]-SA (SA-d_4) (1 mg/0.1 mL MeOH) was dropped onto a filter paper (ϕ 10 mm), and an excess amount of CH_2N_2 in Et_2O was added, and the paper was left for 10 min at room temperature to allow the organic solvent to evaporate. The filter paper was extracted using EtOAc (1 mL) for 1h, and an aliquot (1 mL) of a solution containing MeSA (1 mg/mL in EtOAc) was added to the extract. An aliquot of the mixed solution was subjected to GC-MS analysis as mentioned above. The conversion rate was calculated as following:

conversion rate (%) = (peak area derived from *m/z* 156) x 100 / (peak area derived from *m/z* 152)