Methyl syringate mono-glucoside is a crucial intermediate in leptosperin biosynthesis in *Leptospermum scoparium* (manuka)

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ABSTRACT

Leptosperin (methyl syringate-4-*O*-β-D-gentiobioside) serves as a unique marker for mānuka honey, derived from the manuka plant (*Leptospermum scoparium*). Despite its importance, the biosynthesis pathway of leptosperin remains unreported. This study investigates the molecular mechanism of leptosperin formation from its aglycone, methyl syringate (MSYR), in manuka plants. Methyl syringate-4-*O*-β-D-glucopyranoside (MSYR-glucose) was identified in manuka flower nectar but not in mānuka honey. MSYR was distributed in the flowers, leaves, branches, and roots of manuka plants, while MSYR-glucose and leptosperin were only observed in the flowers. By immersing a cut flowering branch in a deuterium-labeled aqueous medium, the formation of deuterated leptosperin (leptosperin-d_6) and MSYR-glucose (MSYR-d_6-glucose) was analyzed. When MSYR-d_6 was added, both MSYR-d_6-glucose and leptosperin-d_6 were detected. Supplementation with synthetic MSYR-d_6-glucose also generated leptosperin-d_6, indicating that gentiobioside moiety in leptosperin forms through the conjugation of MSYR with D-glucose, followed by the addition of another D-glucose.

Keywords: Mānuka honey, Leptosperin, *Leptospermum scoparium*, Methyl syringate glucosides, Biosynthesis, Certification

INTRODUCTION

Mānuka honey is made from the nectar of the manuka plant (*Leptospermum scoparium*) collected by honeybees. Nectarous dihydroxyacetone (DHA) in the honeycomb (and even in a jar) is partially converted to methylglyoxal (MGO), a known bactericide. In addition, leptosperin (methyl syringate-4-*O*-β-D-gentiobioside), methyl syringate (MSYR), lepteridine, pteridine, phenyllactic acid, and 2′-methoxyacetophenone have also been uniquely or abundantly found in nectar [1-3]. Recently, 19 chemicals were tentatively identified by high-resolution mass spectrometry (MS) as unique compounds in mānuka honey [4]. Manuka foliage (leaf) also contains unique chemicals, such as nortriketones [5]. Among these, leptosperin was exclusively found in *Leptospermum* species, including manuka [6, 7] (Fig. 1). Leptosperin is relatively more stable under prolonged storage and heating than other critical chemicals, such as 2′-methoxyacetophenone and MGO [8]. Owing to its uniqueness to *Leptospermum* honey and its robustness, leptosperin is an essential molecule for Unique Mānuka Factor (UMF) authentication of mānuka honey by the UMF Honey Association. Because the amount of leptosperin is one key element for authentication, the quantification of leptosperin in honey has been achieved by performing MS, fluorescence analysis, or immunochemical techniques, including immunochromatography [1, 2, 6, 9-11]. Given the need to better understand the unique constituents of *Leptospermum* honey, including mānuka honey, this study explored the biosynthetic pathway of one of its key markers, leptosperin.

In the human body, after ingesting mānuka honey, leptosperin is metabolized to MSYR by a bacterial β-glycosidase in the gut [12]. MSYR is further metabolized to syringic acid by carboxylesterase 1 or glucuronate conjugates and MSYR sulfates by phase II enzymes [12, 13]. These metabolites and leptosperin circulate in the blood stream. The metabolism of leptosperin ingested by humans has been gradually elucidated, but not all of it effects, including its possible contribution to biological functions, are known.

Reportedly, the manuka plant has four defined chemical markers (4-hydroxyphenyllactic acid, 4-methoxybenzoic acid, phenyllactic acid, and 2′-methoxyacetophenone) authorized by the New Zealand government [3]. In manuka plants, the levels of the four chemical markers, along with DHA, lepteridine, and leptosperin, increased during flower development [14]. The production mechanism of DHA, a

precursor of MGO, in manuka floral nectar has also been examined [15, 16]. However, the molecular mechanisms underlying biosynthesis of leptosperin and its precursor in manuka plants has not been reported. Since mānuka honey is often disguised, knowledge of leptosperin biosynthesis could strengthen the robustness of mānuka honey authentication and be useful for consumer protection.

The study aim was to investigate how MSYR-4-*O*-β-D-glucopyranoside (MSYR-glucose), a key intermediate in leptosperin biosynthesis and a component of manuka nectar, is produced and further metabolized by manuka plants. We used stable isotopic template molecules to show that MSYR-glucose was primarily generated from MSYR, and leptosperin was then constructed from MSYR-glucose with another glucose.

MATERIALS AND METHODS

Materials

Tetra-*O*-acetyl-α-D-glucopyranosyl bromide and forchlorfenuron (FCF) were obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Gentiobiose octaacetate was purchased from Carbosynth, Ltd. (Staad, Switzerland). CD_3I was obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Tetrabutylammonium bromide was a product of Kishida Chemical Co. Ltd. (Osaka, Japan). MSYR was purchased from Alfa Aesar (Ward Hill, MA). Sodium methoxide (1 mol/L) was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Dimethyl sulfoxide (DMSO) and formic acid (FA) were purchased from Fujifilm Wako Chemical Corporation (Osaka, Japan). Leptosperin was chemically synthesized according to a previously reported method [17]. Samples of mānuka honey (Manuka South, 100% Pure New Zealand Honey, and Honey Valley) were obtained from Manuka South and Green Bay Co.

Synthesis of MSYR-d_6, MSYR-glucose, MSYR-d_6-glucose and leptosperin-d_6

A phase-transfer catalyst was used to synthesize MSYR-glucose [18]. To a reaction mixture containing MSYR (146 mg), tetra-*O*-acetyl-α-D-glucopyranosyl bromide (290 mg), and tetrabutylammonium bromide (290 mg) in 15 mL of $CHCl_3$, 10 mL of NaOH (1 mol/L) was added and stirred vigorously at ambient temperature for 5 h. The reaction mixture was dissolved in 200 mL ethyl acetate and washed with 5% Na_2CO_3, water, and saturated brine. After drying over anhydrous Na_2SO_4, the extract was concentrated under vacuum. The concentrate was then separated by elution using a solvent mixture of hexane/ethyl acetate (2:3) on two 1-mm-thick Merck silica gel plates. The acetylated product (166 mg) was reacted with 1.2 mL sodium methoxide in 10 mL CH_3OH. After 15 min, the solution was applied to an Amberlite® IR120B (Organo Corp., Tokyo, Japan) column (15 × 280 mm) and eluted with 300 mL of CH_3OH. The MSYR-glucose was obtained after concentration *in vacuo* (47% from MSYR). The product was purified by preparative high-performance liquid chromatography (HPLC) on a Wakosil-II 5C18HG column (20 × 250 mm, Wako Pure Chemical Industries), with an elution solvent mixture containing water/CH_3OH (7:3) at a 5.0 mL/min flow rate at ambient temperature. The ^{13}C nuclear magnetic resonance (NMR) spectrum was obtained on a Bruker AVANCEIII400HD spectrometer using the solvent peak as the

internal standard (δ_C 150.0 ppm) and showed good agreement with previous ^{13}C NMR spectra [19]. Combined with the mass spectra, the structure, including the β-glucoside bond, was confirmed as $[M+NH_4]^+$: Theoretical for $C_{16}H_{26}O_{10}N$ 392.1551, found 392.1544; $[M+FA–H]^–$: Theoretical for $C_{17}H_{23}O_{12}$ 419.1195, found 419.1197.

For MSYR-d_6-glucose and leptosperin-d_6 synthesis, MSYR-d_6 was prepared as described previously [17], except that CD_3I was used. The mass spectra ($[M+H]^+$: Theoretical for $C_{10}H_7D_6O_5$ 219.1134, found 219.1108; $[M–H]^–$: Theoretical for $C_{10}H_5D_6O_5$ 217.0989; found 217.0991) suggested that this product contains six deuterium atoms. Moreover, a signal at δ_H 7.32 ppm (s, 2H) on the ^1H NMR spectrum ($CDCl_3$) suggested a symmetrical product structure. Two hexa-deuterated samples, MSYR-d_6-glucose and leptosperin-d_6, were prepared by conjugating MYSR-d_6 to tetra-*O*-acetyl-α-D-glucopyranosyl bromide or to hepta-*O*-acetyl-α-gentiobiosyl bromide, which was prepared from gentiobiose octaacetate [20]. The products were purified in a manner similar to that for leptosperin [7] and MSYR-glucose.

The following MS results were obtained: MSYR-d_6-glucose ($[M+NH_4]^+$: Theoretical for $C_{16}H_{20}D_6O_{10}N$ 398.1928, found 398.1934; $[M+FA–H]^–$: Theoretical for $C_{17}H_{17}D_6O_{12}$ 425.1572, found 419.1575). Leptosperin-d_6 ($[M+NH_4]^+$: Theoretical for $C_{22}H_{30}D_6O_{15}N$ 560.2456, found 560.2462; $[M+FA–H]^–$: Theoretical for $C_{23}H_{27}D_6O_{17}$ 587.2100, found 587.2101).

Sample preparation

Manuka (*Leptospermum scoparium*) plants were purchased from retail stores (garden stores). Nectar was repeatedly collected from several flowers by pipetting with 50 µL of water. A centrifugal filtration apparatus (Sartorius, Vivaclear Mini, 0.8 µm) was then used to filter the obtained aqueous nectar. The flowers (with pistil, petal, and sepal), leaves, and branches (n = 3) were collected, weighed, and transferred to a tube containing beads (Lysing Matrix A). The solvent (methanol/water = 1/1) (0.1 g sample/mL) was added to the sample, and the tube contents were homogenized for 40 sec at 6.0 m/sec on a Fast Prep 24 5G (MP-Biomedicals). The tube was then centrifuged to remove debris and beads. The supernatant was collected and filtered, as previously described.

The mānuka honey was diluted to 0.1 g/mL in water, centrifuged, and the supernatant analyzed as described in the HPLC-fluorescence analysis section.

Experiment with MSYR-d_6 or MSYR-d_6-glucose supply

A branch (approximately 5 cm) with three flowers was cut, and the edge of the branch was immersed in a tube containing 10% dimethyl sulfoxide (DMSO) in water containing 1 mM MSYR-d_6 or MSYR-d_6-glucose. After 24 h at room temperature, the flowers were collected and homogenized. The samples were then filtered as described previously.

HPLC-fluorescence analysis

HPLC (Shimadzu Prominence) connected to a fluorescence detector (RF-10AXL) was performed to separate and identify the chemical components in aqueous samples from nectar or mānuka honey. The separation was performed on a Kinetex XB-C18 column (5.0 µm, 4.6 × 150 mm, Phenomenex) by gradient elution with 0.1% FA in water (solvent A) and acetonitrile (solvent B) at a 1.0 mL/min flow rate. The detector was set to an excitation wavelength of 267 nm and an emission wavelength of 362 nm. The linear gradient program was as follows: 0 min (10% B), 10 min (40% B), 11 min (10% B), and 22 min (10% B).

Ultra-HPLC quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF MS) analysis

The filtrate was mixed with an equal volume of 10 ng/mL FCF as an internal standard. Standards containing MSYR, MSYR-d_6, MSYR-glucose, MSYR-d_6-glucose, leptosperin, and leptosperin-d_6 (Fig. 1) were mixed with the internal standard to generate a standard curve. The sample was separated on an Exion UHPLC instrument (Sciex) with a Kinetex XB-C18 column (2.6 µm, 2.1 × 100 mm) and gradient elution. The mobile phase was 0.1% FA in water (solvent A) and methanol (solvent B), and the flow rate was 0.4 mL/min. The linear gradient program was as follows: initial 0% B, 4.5 min 100% B, 5 min 0% B, and hold for 5min before the next injection. The eluate was introduced into a Q-TOF mass spectrometer (SCIEX X500R). Information-dependent analysis (IDA), high-resolution multiple-reaction monitoring (MRM^{HR}), and product ion scans in positive or negative modes were performed. The combinations of MRM^{HR} and TOF-MS are listed in the Supplementary Materials (Tables S-1 and S-2). Negative

MRM was used to quantify the glucosides, and positive MRM was performed for the aglycones MSYR/MSYR-d_6.

Statistical analysis

Unless otherwise indicated, all experiments were performed on $n = 3$ samples, and the quantitative results are expressed as the mean ± standard deviation.

RESULTS

Identification of MSYR-glucose in the nectar of manuka plants

Leptosperin has unique fluorescence characteristics [2, 9], and sensitive leptosperin detection from the nectar of manuka flowers was accomplished during chromatographic separation with a fluorescence detector (excitation = 267 nm/emission = 362 nm). As shown in Fig. 2, two peaks were observed for the nectar sample. The elution time (7.3 min) of the earlier peak was identical to that of the authentic leptosperin. The later peak was assumed to be MSYR-4-*O*-β-D-glucopyranoside (MSYR-glucose) because the retention time (8.5 min) matched that of the chemically synthesized MSYR-glucose. Leptosperin has previously been found in manuka flower nectar and mānuka honey [2, 3] (Fig. 2), but there have been no reports of MSYR-glucose in nectar or honey. To further confirm this result, the nectar sample was separated and analyzed by UHPLC-Q-TOF. We observe signals for $[M+NH_4]^+$ at 392.1559 (theoretical 392.1551 for MSYR-glucose) with positive ionization and for $[M+FA–H]^-$ at 419.1193 (theoretical 419.1195) with negative ionization of the compound. The positive/negative fragmentation patterns of the peak matched the fragmentation pattern of the synthetic MSYR-glucose (Fig. S1), and the predicted fragmentations from the chemical structure of MSYR-glucose (data not shown). On the basis of these results, this compound was identified as MSYR-glucose. The MSYR-glucose in the nectar was 40% of the intensity of nectary leptosperin (Fig. 2). Because the fluorescence properties of MSYR-glucose were the same as those of leptosperin (data not shown), the peak intensity reflected the amount. From a structural point of view, the presence of MSYR-glucose in the nectar of manuka could indicate the participation of the mono-glucoside in the leptosperin biosynthesis.

Distribution of unique MSYR-related chemicals in manuka plants

The distribution of MSYR-related chemicals in manuka plants was examined. The flowers, leaves, roots, and branches were separately homogenized, and MSYR, MSYR-glucose, and leptosperin contents were quantified. Typical MRM^{HR} chromatograms are shown in Fig. 3A. Two MRM^{HR} transitions for one molecule were monitored to identify individual molecules. Leptosperin and MSYR-glucose were observed at 2.78 min and 3.10 min, respectively. Notably, when monitoring MSYR, the

sample peak matched the standard MSYR (3.85 min), and two earlier additional peaks (2.7 and 3.15 min) were observed. The earlier peaks originated from the fragmentation of MSYR-glucose and leptosperin to MSYR during the ionization process. MSYR (approx. 0.2–1 nmol/g tissue) was detected in all manuka parts examined. MSYR-glucose and leptosperin were more abundant in flowers but not detected in the leaves, roots, and branches under the experimental conditions (Fig. 3B). The MSYR-glucose amount in the flowers was 6.5 times higher than the leptosperin amount, which was the opposite of the nectar amount (Fig. 2).

Biosynthesis of deuterium leptosperin and MSYR-glucose from MSYR-d_6

To distinguish naturally presented MSYR, we chemically synthesized MSYR-d_6. MSYR-d_6 (1 mM) was then added to a cross section of branches with manuka flowers. After 24 h, the flowers were homogenized, and the MSYR-related chemicals with six deuterium atoms were analyzed as described in the Materials and Methods. Signals for MSYR-d_6-glucose and leptosperin-d_6 were observed (Fig. 4A, B). The fragmentation patterns of deuterium glucosides, MSYR-d_6-glucose, and leptosperin-d_6 indicated liberation of the MSYR-d_6 moiety ($[M–H]^-$ 217) by collision-induced dissociation (Fig. 4A, C).

The presence of MYSR-d_6 (approximately 800 nmol/g flower) indicated that the supplemented MSYR-d_6 was transported to flowers via the phloem (Fig. 5A, open bar). Simultaneously, MSYR-d_6-glucose (approximately 200 nmol/g flower; Fig. 5B, filled bar) and leptosperin-d_6 (approx. 5.3 nmol/g flower; Fig. 5C, right bar) were detected in the flowers. These findings suggest that MYSR-glucose is synthesized from MSYR. Leptosperin was formed by conjugating MSYR-glucose with additional glucose and/or by conjugating MSYR with gentiobiose (diglucoside).

MSYR-d_6-glucose supplemented generates the deuterium leptosperin

To confirm further conjugation of MSYR-glucose with additional glucose, MSYR-d_6-glucose was supplemented, and the formation of leptosperin-d_6 in the flower was analyzed. As shown in Fig. 4C, the leptosperin-d_6 signal was observed at 2.7 min (Fig. 4C, c). Detection of supplemented MSYR-d_6-glucose in the flower (approximately 450 nmol/g flower) indicated that significant amounts of MSYR-d_6-glucose were transported to the flower (Fig. 5B, open bar). Formation of leptosperin-d_6 (approx. 5.5

nmol/g flower) was observed after MSYR-d_6-glucose supplementation (Fig. 5C, right bar), which was approximately five-fold higher than that after MSYR-d_6 supplementation (Fig. 5C, left bar). These findings indicate at least one pathway of leptosperin synthesis from MSYR-glucose with additional glucose. Interestingly, when MSYR-d_6-glucose was added, the cleaved product MSYR-d_6 was also observed (Fig. 5A, right bar), which suggests that MSYR-glucose can be partly cleaved into MSYR by glycosidase.

DISCUSSION

Mānuka honey has high antibacterial activity owing to MGO, which originates from plant-derived DHA [21, 22], and is thought to also have anti-inflammatory effects, among others. Owing to its scarcity and expected functionalities, mānuka honey is expensive, which could lead to marketing of honey that is made to look like mānuka honey. To certify "genuine" mānuka honey, leptosperin was used as one marker because it is exclusively found in the mānuka honey [6, 7]. However, the reason for the uniqueness of leptosperin remains unclear. In this study, we found MSYR-glucose in nectar and manuka flowers. MSYR-glucose has been identified from anis (*Pimpinella anisum*) [19], but there are no reports of MSYR-glucose being found in mānuka honey or the plant. From a structural perspective, MSYR-glucose could be an intermediate of leptosperin, a diglucoside of MSYR (Fig. 1). Because presence of leptosperin has already been included in the UMF certification of mānuka honey, knowledge of the biosynthesis of leptosperin in manuka plants is vital to strengthen the certification. In addition, the lack of MSYR-glucose in mānuka honey (Fig. 2) is interesting because MSYR-glucose is abundant in plant nectar of manuka (Fig. 3B). This discrepancy in MSYR-glucose is currently under investigation and is briefly discussed in this section. From an applied perspective, if MSYR-glucose is abundant in mānuka honey, it is possible that manuka flowers or pollen were artificially added to increase the content of leptosperin, which is an important element in the certification/grading system of mānuka honey.

The distribution of the MSYR-related chemicals was also examined. MSYR was found in the flowers, leaves, roots, and branches (Fig. 3). Conversely, MSYR-glucose and leptosperin were found in flowers, but were below the detection limits in the other parts examined. These findings suggest that leptosperin and MSYR-glucose are synthesized in the flowers.

We observed serial conjugations of glucose with MSYR to build MSYR-glucose and then leptosperin. Notably, the tri-glucoside of MSYR was also detected [4]. Plant glucosides are often synthesized by the 1 UDP-glucosyltransferase (UGT/GT1) family [23, 24]. Reportedly, quercetin 3-*O*-gentiobioside and curcumin-4′-*O*-gentiobioside are generated from their respective mono-glucosides by CaUGT3 (*Catharanthus roseus* glycosyltransferase 3) [25, 26]. The enzyme responsible for the glucosylation of MSYR remains to be elucidated. A whole-genome assembly of *Leptospermum scoparium* has

previously been shown [27], and information on this gene could be beneficial for future studies on enzyme identification.

As mentioned, leptosperin is a unique molecule found in manuka plants and honey. MSYR is rich in mānuka honey but is also observed in asphodel honey and Zantaz (*Bupleurum spinosum*) honey [28, 29]. We have previously confirmed that asphodel honey does not contain leptosperin [7]. Additionally, we have found that the tea tree (*Melaleuca alternifolia*), which belongs to the same Myrtaceae (subfamily Leptospermoideae) as manuka, has MSYR and MSYR-glucose, but not leptosperin (unpublished observation). There are two possible reasons for the uniqueness of leptosperin: 1) MSYR is not a common phytochemical, and 2) the enzyme (glucosyltransferase) is unique that has not yet been identified.

In this study, MSYR-glucose was identified for the first time in mānuka nectar. MSYR-glucose was more than six-fold richer than leptosperin in flowers (Fig. 3) but only approximately one-third of leptosperin in the nectar (Fig 2). Leptosperin is abundant in mānuka honey, but MSYR-glucose is not. A high-sensitivity mass spectrometer detected only trace MSYR-glucose in mānuka honey (unpublished observation), which suggests that an enzyme from the nectar or honeybee stomach (including microbes) cleaves the glucoside bond between MSYR and the glucose of MSYR-glucose, but not the bonds in leptosperin, during honey maturation. This possibility is currently being investigated.

We confirmed the assembly of glucosides to synthesize the unique chemical leptosperin from a chemical perspective, but this study has some drawbacks. First, the enzyme that mediates glucosylation has not yet been identified. Second, the reason for the discrepancy in the ratio of MSYR-glucose to leptosperin between the flower and nectar is unknown.

In summary, we identified MSYR-glucose in nectar and in flowers of the manuka plants. Stable isotopic MSYR and MSYR-glucose were prepared and used to study the dynamics of the native chemicals. MSYR-glucose was found to have an essential role as a critical intermediate in leptosperin biosynthesis. However, the key enzyme is unknown, but a future investigation might demonstrate the uniqueness of the manuka plant and strengthen the authentication of mānuka honey performed by measuring its leptosperin content.

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ABBREVIATIONS

MSYR, methyl syringate; DHA, dihydroxyacetone; MGO, methylglyoxal; UMFHA, Unique Mānuka Factor Honey Association; FCF, forchlorfenuron; DMSO, dimethyl sulfoxide; FA, formic acid; Q-TOF/MS, quadrupole time-of-flight high-resolution mass spectrometry; IDA, information-dependent analysis; MRM^{HR}, high-resolution multiple-reaction monitoring

353 **Figure legends**

354

355 **Figure 1.** Chemical structures of methyl syringate (MSYR)-related chemicals.
356 Left: native (natural) MSYR, MSYR-glucose, and leptosperin. Right: deuterium-labeled
357 MSYR, MSYR-glucose, and leptosperin. Arrows indicate the direction of biosynthesis.

358

359 **Figure 2.** HPLC-fluorescent detection of manuka nectar and honey. Manuka honey and
360 manuka nectar were analyzed by HPLC. Top left: Standard leptosperin. Top right:
361 Standard MSYR-glucose. Left below: Mānuka honey. Right below: Manuka nectar.

362

363 **Figure 3.** Distribution of natural methyl syringate (MSYR)-related phytochemicals.
364 Plant tissues were homogenized and analyzed as described in the Materials and
365 Methods. (A) Typical MRM^{HR} chromatograms of manuka flower homogenate. (B)
366 Quantification of MSYR-related phytochemicals in plant tissues.

367

368 **Figure 4.** Identification of methyl syringate (MSYR)-d_6-glucose and leptosperin-d_6
369 biosynthesis in manuka plants. MSYR-d_6 was supplemented and analyzed (A, B, a, b).
370 MSYR-d_6-glucose was supplemented and analyzed (C, c). MSYR-d_6-glucose was
371 tracked by monitoring the extracted ion at 425, corresponding to the $[M+FA–H]^-$ of
372 MSYR-d_6-glucose in TOF/MS (A). Leptosperin-d_6 was tracked by monitoring the
373 extraction ion at 587, corresponding to $[M+FA–H]^-$ of leptosperin-d_6, from TOF/MS (B,
374 C). The product ion scans of the respective precursors (425 or 587) and the chemical
375 structures with the possible cleaved sites for the generation of fragment 217 are shown
376 (a, b, c).

377

378 **Figure 5.** Quantitation of deuterium glucosides in flowers supplemented with methyl
379 syringate (MSYR)-d_6 or MSYR-d_6-glucose. The cut edge of the plant branch was
380 immersed in aqueous water containing 1 mM MSYR-d_6 or MSYR-d_6-glucose. "MSYR-
381 d_6 +" and "MSYR-d_6-Glc +" mean the supplementation of MSYR-d_6 and of MSYR-d_6-
382 glucose (1 mM), respectively. Three chemicals with six deuterium atoms were analyzed
383 and quantified. (A) MSYR-d_6. (B) MSYR-d_6-glucose. (C) Leptosperin-d_6. An open bar
384 indicates the quantitative results of the same chemicals as those supplemented,

indicating that the chemical was transferred to the tissue. The filled bars indicate the formation of newly biosynthesized deuterium-containing compounds.

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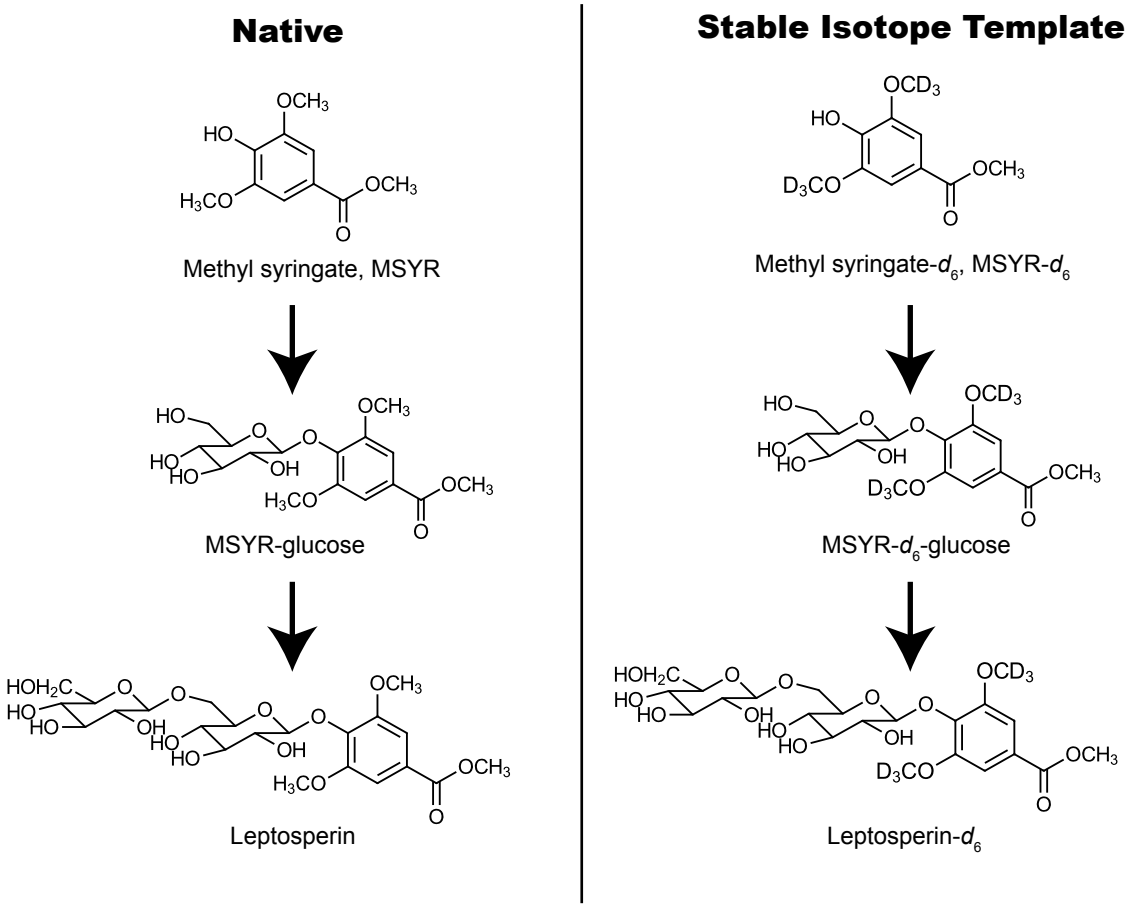
494 **Figure 1**

495



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498 **Figure 2**

499

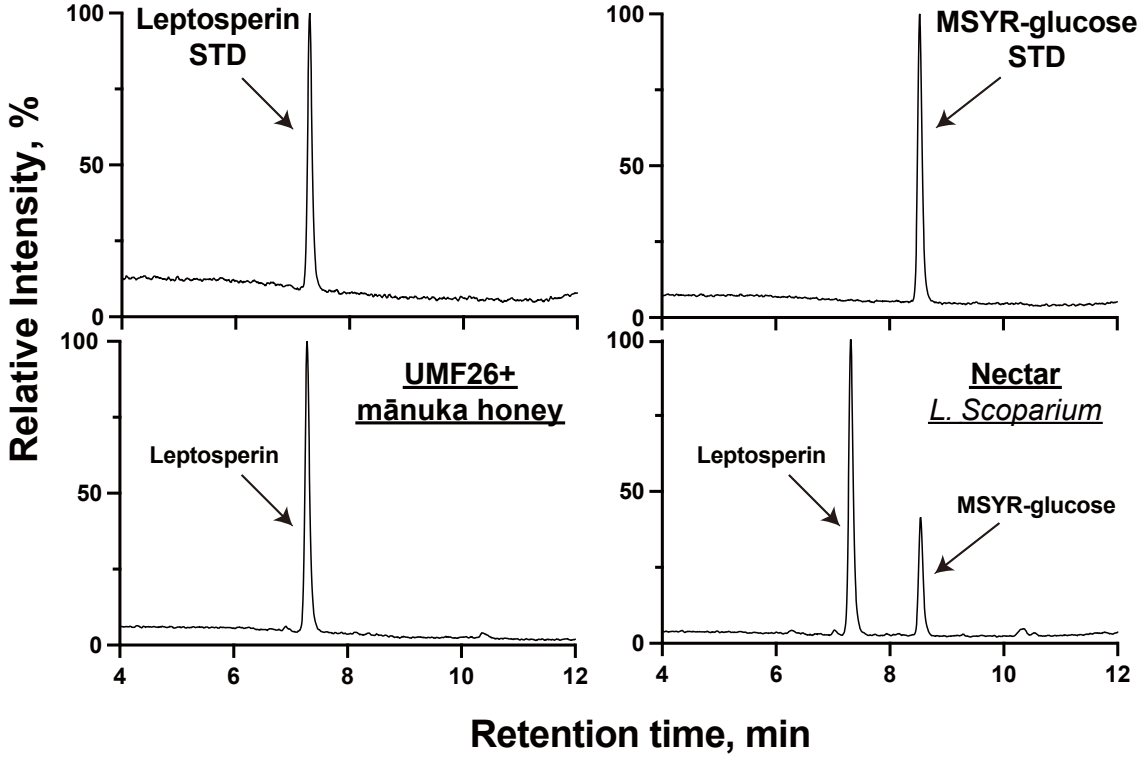

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Figure 3

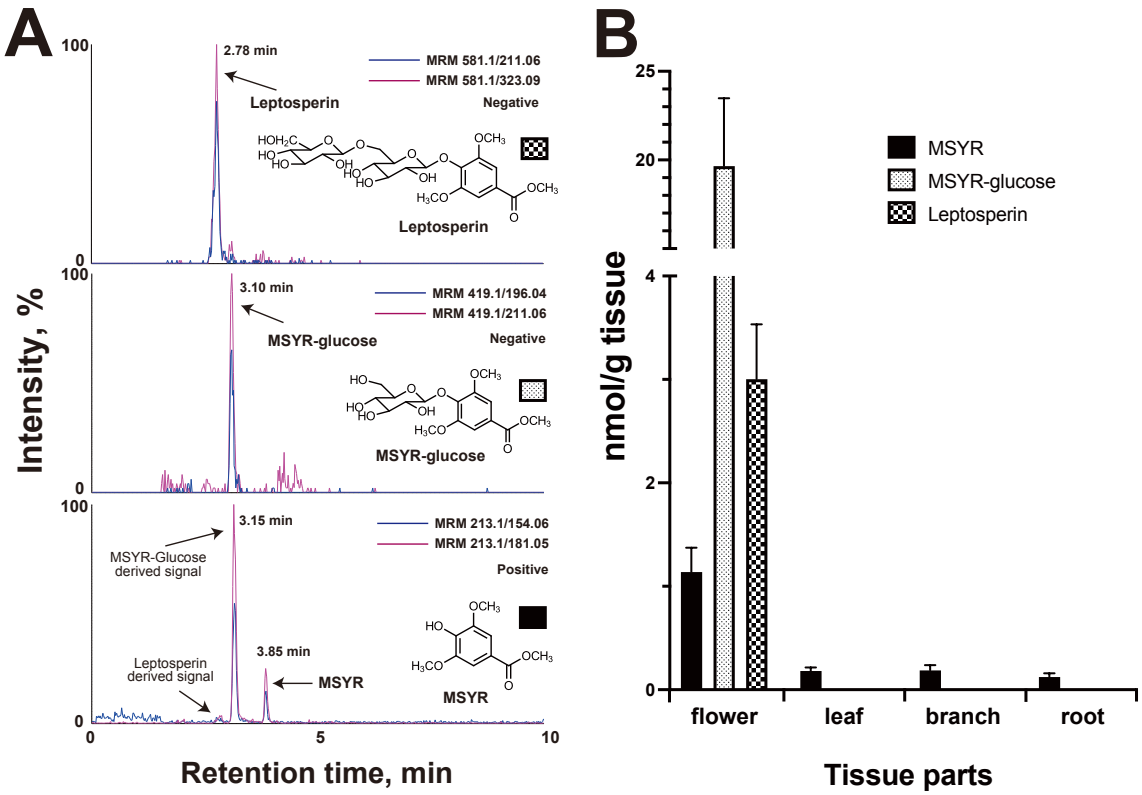

A
100
0
2.78 min
MRM 581.1/211.06
MRM 581.1/323.09
Negative
Leptosperin
Leptosperin
3.10 min
MRM 419.1/196.04
MRM 419.1/211.06
Negative
MSYR-glucose
MSYR-glucose
3.15 min
MRM 213.1/154.06
MRM 213.1/181.05
Positive
MSYR-Glucose derived signal
Leptosperin derived signal
3.85 min
MSYR
MSYR
Intensity, %
0
5
10
Retention time, min
B
25
20
4
2
0
MSYR
MSYR-glucose
Leptosperin
nmol/g tissue
flower
leaf
branch
root
Tissue parts

Figure 4

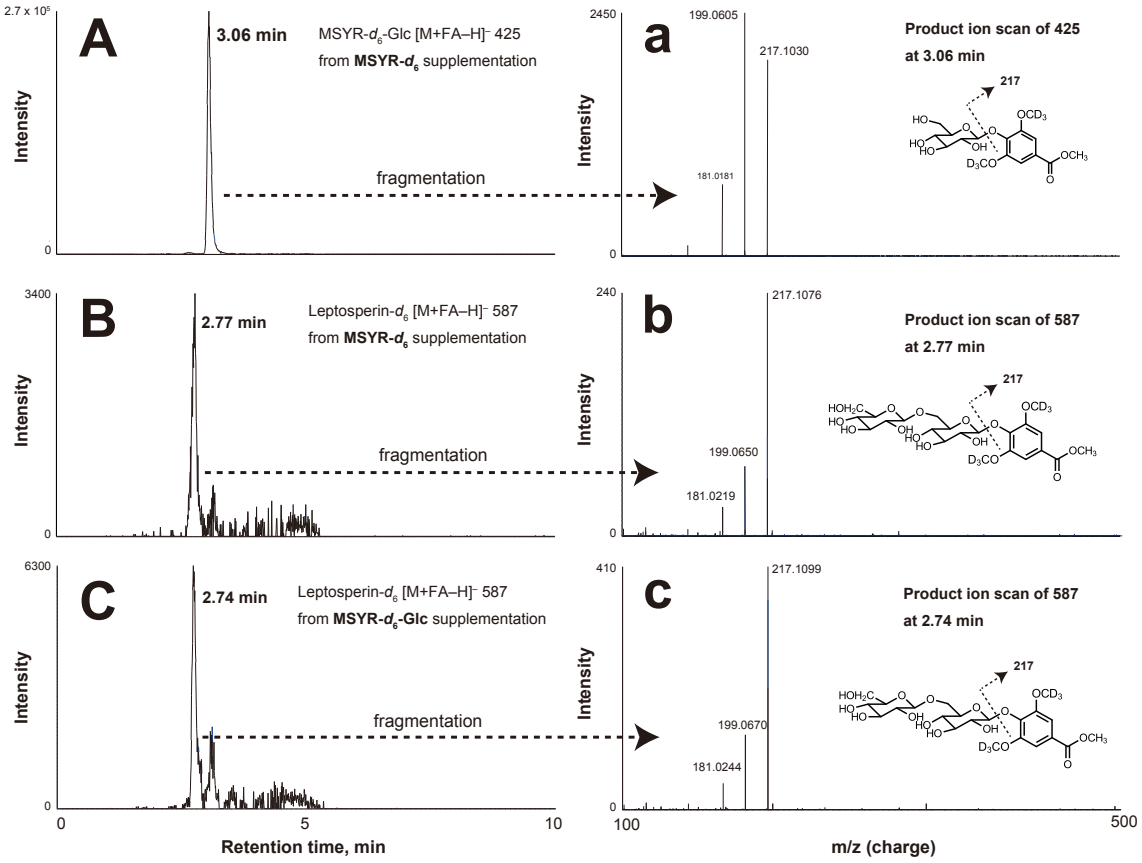
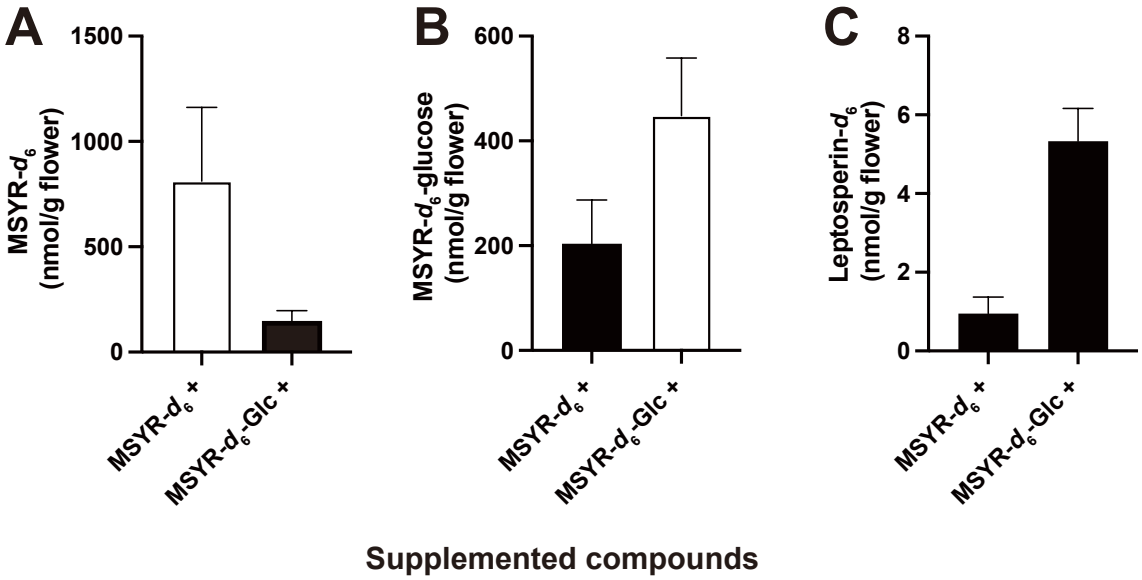
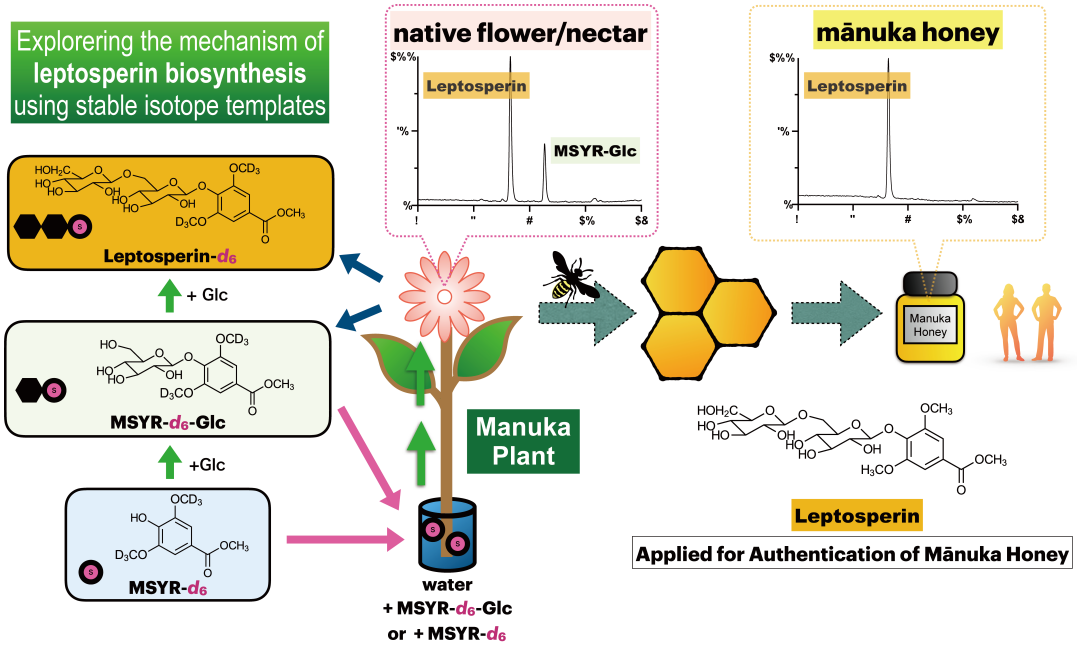

A
3.06 min
MSYR-d_6-Glc [M+FA–H]$^-$ 425
from **MSYR-d_6** supplementation
fragmentation
a
199.0605
217.1030
181.0181
Product ion scan of 425
at 3.06 min
B
2.77 min
Leptosperin-d_6 [M+FA–H]$^-$ 587
from **MSYR-d_6** supplementation
fragmentation
b
217.1076
199.0650
181.0219
Product ion scan of 587
at 2.77 min
C
2.74 min
Leptosperin-d_6 [M+FA–H]$^-$ 587
from **MSYR-d_6-Glc** supplementation
fragmentation
c
217.1099
199.0670
181.0244
Product ion scan of 587
at 2.74 min
Intensity
Retention time, min
m/z (charge)

Figure 5


A
MSYR-d_6 (nmol/g flower)
1500
1000
500
0
MSYR-d_6 +
MSYR-d_6-Glc +
B
MSYR-d_6-glucose (nmol/g flower)
600
400
200
0
MSYR-d_6 +
MSYR-d_6-Glc +
C
Leptosperin-d_6 (nmol/g flower)
8
6
4
2
0
MSYR-d_6 +
MSYR-d_6-Glc +
Supplemented compounds

515 Graphical Abstract



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