Running title: Glycosidically bound precursors of damascenone

Title: Formation of damascenone derived from glycosidically bound precursors in green tea infusions

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Abstract

Damascenone is well-known for its potent flavor with an extremely low odor threshold. Several glycosidically bound precursors of damascenone have been isolated from several plants, but little is known about their occurrences in green tea infusions. In this work, three major glycosidic precursors of damascenone, 9-*O*-β-D-glucopyranosyl-megastigma

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-6,7-dien-3,5,9-triol (**1a**), 9-*O*-β-D-glucopyranosyl-3-hydroxy-7,8-didehydro-β-ionol (**2a**), and 3-*O*-β-D-glucopyranosyl-3-hydroxy-7,8-didehydro- β-ionol (**2b**) were isolated and identified in green tea infusions, and the stereochemistries at C-3 and C-9 positions of aglycone parts of the three glycosidic precursors were determined as (3*S*, 9*R*)-**1a**, (3*R*, 9*R*)-**2a**, and (3*R*, 9*R*)-**2b**, respectively. Compounds **1a**, **2a**, and **2b** as well as 3-*O*-β-D-glucopyranosyl-megastigma-6,7-dien-3,5,9-triol (**1b**) were hydrolyzed to form damascenone in a model system with strong acidic conditions (pH 2.0) and at high temperature (90°C). In contrast to hydrolysis of **2a** and **2b**, more damascenone was transformed from **1a** and **1b**. Furthermore, the β-D-glucosyl moiety at C-3 position gave a higher dehydration rate from megastigma-6,7-dien-3,5,9-triol to 3-hydroxy-7,8-didehydro-β-ionol than compound **1a** carrying the sugar residue at C-9 position. Interestingly, the four glycosidic precursors of damascenone were not hydrolyzed to give damascenone under slightly acidic conditions (pH 5.4 and 120°C for 10 min), but they could be transformed to damascenone in the presence of green tea infusions even under the equal conditions.

Keywords: Damascenone; Green tea; Glycosides; Precursor

Introduction

Damascenone is a potent flavor compound, possessing an extremely low odor threshold of 0.002 ppb in water (**Buttery, Teranishi, Ling, & Turnbaugh, 1990**). In addition to Bulgarian rose (*Rosa damascena*) oil, where it was firstly identified (**Demole, Enggist, Säuberli, Stoll, & Kováts, 1970**), damascenone or its precursors are found in other natural products such as tomatoes (**Buttery, Teranishi, & Ling, 1987**), apples (**Roberts, Morehai, & Acree, 1994**), grapes (**Shure, & Acree, 1994**), and in plant-derived beverages such as beers (**Chevance,**

45 **Guyot-Declerck, Dupont, & Collin, 2002**), wines (**Kotseridis, Baumes, & Skouroumounis,**
46 **1999**), and black teas (**Kumazawa, & Masuda, 2001**). Several glycoconjugates of the two
47 polyol precursors of damascenone, 9-*O*-β-D-glucopyranosyl-megastigma-6,7-dien-3,5,9-triol
48 (**1a**), 3-*O*-β-D-glucopyranosyl-megastigma-6,7-dien-3,5,9-triol (**1b**),
49 9-*O*-β-D-glucopyranosyl-3-hydroxy-7,8-didehydro-β-ionol (**2a**), and 3-*O*-β-D-
50 glucopyranosyl-3-hydroxy-7,8-didehydro-β-ionol (**2b**), have been isolated from various plant
51 tissues such as *Lycium halimifolium* (**Näf, Velluz, & Thommen, 1990**), apples (**Roberts,**
52 **Morehai, & Acree, 1994**), *Rosa damascena* Mill. (**Straubinger, Knapp, Oka, Watanabe, &**
53 **Winterhalter, 1997; Suzuki et al., 2002**), and Riesling wine (**Baderschneider, Skouroumounis,**
54 **& Winterhalter, 1997**), demonstrating the importance of stored glycosides as precursors of
55 damascenone. Volatile norisoprenoids can be released by acid-catalyzed transformations from
56 their glycocunjugates (**Skouroumounis, & Sefton, 2000; Cox et al., 2005**).
57 Damascenone was determined as one of the most important odor components of Japanese
58 green tea (Sencha) products (**Kumazawa, & Masuda, 1999**), and increased in concentration
59 during the processing of black tea infusions (**Kumazawa, & Masuda, 2001**). Although it was
60 proposed that precursors of damascenone in the black tea infusions may be glycosides of
61 3-hydroxy-7,8-didehydro-β-ionol (**2**) by enzymatic reaction from a crude glycoside fraction
62 obtained from green tea infusions (**Kumazawa, & Masuda, 2001**), the chemical structure was
63 not completely elucidated. In this work, we attempt to confirm presence of glycosidic
64 precursors of damascenone in green tea infusions. We elucidated chemical structures of all
65 glycosidic precursors including the stereochemistries at C-3 and C-9 positions of the aglycone
66 moieties on the basis of high performance liquid chromatography-mass spectrometry (HPLC-MS)
67 and nuclear magnetic resonance (NMR) spectroscopy. In addition, we also investigated

transformation of each glycosidic precursor (**1a**, **1b**, **2a**, and **2b**) to damascenone under acidic conditions and elevated temperatures.

Materials and methods

Isolation and purification of the glycosidic precursors of damascenone from green tea infusions, and HPLC-MS and NMR analysis (See a brief scheme in Figure1)

Dried green tea (*Camellia sinensis* var. Assamica) leaves were kindly provided by Mitsui Norin Co., Ltd., Japan, extracted with boiled water and spray-dried to powder. One hundred grams of the extraction powder of green tea were dissolved in 600 mL of distilled water, and extracted with $CHCl_3$ (100 mL × 5). One hundred grams of Polyclar AT were added to the residual aqueous fraction to remove polyphenols, and the resultant mix was centrifuged at 3000 *g* for 15 min. One hundred grams of Polyclar AT were added to this supernatant filled up with distilled water to a volume of 600 mL and centrifuged again as described above. The final supernatant was diluted with 8500 mL of water and then subjected to chromatography on Amberlite XAD-2 in a 20 × 8 cm (i.d.) glass column. The column was eluted with 500 mL of distilled water to give Fraction I, 750 mL of 50% aqueous acetone to give Fraction II, and 1000 mL of 100% acetone to obtain Fraction III, successively. Damascenone was only detected from conversion of Fraction II in a model system with strong acidic (pH 2.0) and high temperature (90°C) conditions (data not shown), suggesting that Fraction II contained the glycosidic precursors of damascenone. After concentration, Fraction II was acetylated (0.37 M of pyridine-0.32 M of acetic anhydride) and purified using silica gel C200 (100 g) column (12 × 4.5 cm i.d.) chromatography with a solvent gradient of 20%→30%→40%→50% →60%→70% ethyl acetate in hexane to obtain six fractions (Fraction II -1 ~ Fraction II -6). To detect the

glycosidic precursors of damascenone, each fraction was deacetylated by treating with 1 M of sodium methoxide at room temperature overnight, neutralized with Dowex 50W-X4 (H^+-form), and then lyophilized. Damascenone was released from the deacetylated fractions (Fraction II-2~ Fraction II-5) in the model system as described above (data not shown).

The Fractions II-2 and II-3 were combined and subjected to silica gel C200 (20 g) column (18 × 2.3 cm i.d.) chromatography with a solvent gradient of 20%→30%→40%→50% ethyl acetate in hexane to give six fractions 1a-6a. From the 6 fractions, fraction 3a was purified by a silicagel $60F_{254}$ preparative TLC [plate: 20 × 20 cm; Solvent: 80% butyl methyl ether (TBME) in hexane] to give nine fractions 1b-9b. From the 9 fractions, fraction 4b was deacetylated as described above, and then further purified by HPLC using an ODS YMC-pack-AM column (4.6 mm× 250 mm) with a linear gradient from 10% to 30% acetonitrile in 20 min at 40°C and a detection wavelength of 230 nm to obtain seven fractions 1c-7c. From the 7 fractions, fractions 3c (Fraction II-A) and 4c (Fraction II-B) were analyzed by a HPLC (Waters 2690)-MS (Thermo quest TSQ 7000) equipped with a 2.0 mm × 150 mm i.d., Develosil ODS HG-3 column, respectively. The sample solutions were chromatographed using gradient elution with water as solvent A and methanol as solvent B, at a flow rate of 0.2 mL/min at 40°C. Elution was started under isocratic conditions of 5% of solvent B for 10 min, and followed by a linear increase of solvent B to 70% at 30 min. Positive ionization mode was applied in SIM scanning mode at *m/z* 393, 409, 411, and 427. Optimized electrospray operating conditions were: spray voltage 4.5 kV, heated carrier temperature 350 ºC, sheath gas N_2 (80 psi), and auxiliary gas N_2 (40 psi). For analysis of chiral glycosidic precursors, the Fraction II-B was further determined by HPLC equipped with a 4.6 mm × 250 mm, Chiralpak AD-H column using a linear gradient from 10% to 90% 2-propanol in hexane in 60 min at 40°C, 1 mL/min of flow rate, and at a detection

wavelength of 230 nm.

The Fractions II-4 and II-5 were combined and subjected to silica gel C200 (100 g) column (30 × 3.1 cm i.d.) chromatography with a solvent gradient of 40%→50% →60%→70% ethyl acetate in hexane to give six fractions 1a′-6a′. From the 6 fractions, fraction 2a′ was deacetylated as described above, and then fractionated by HPLC using an ODS YMC-pack-AM column (20 mm× 250 mm) with a linear gradient from 10% to 60% acetonitrile in 40 min at 40°C and at a detection wavelength of 220 nm to obtain five fractions 1b′-5b′. From the 5 fractions, fractions 2b′ and 3b′ were further fractionated into 3 fractions 1c′-3c′ and 1c′′-3c′′, respectively, by HPLC using an J'sphere ODS H-80 column (4.6 mm× 150 mm) with a linear gradient from 5% to 25% acetonitrile in 20 min at 40°C and at a detection wavelength of 220 nm. The obtained fractions 2c′ and 2c′′ (Fraction II-C) were further purified by HPLC again under equal conditions to give a purified glycosidic precursor, which was identified by ^{1}H-NMR analyses (JEOL JNM-LA 500 NMR spectrometer).

Hydrolysis of glycosidic precursors to damascenone

The glycosidic precursors (**1a**, **1b**, **2a**, and **2b**) including their chiral compounds were synthesized according to the method by Yamano et al. (**Yamano, Watanabe, Watanabe, & Ito, 2002**). Hydrolysis of each glycosidic precursor (10 μg) to damascenone with or without the presence of green tea infusions (equal to 1 g of the boiled water extract of tea leaves) were investigated under a similar condition (pH 5.4 and 120°C for 10 min) which occur during the sterilization process of bottled and canned green tea drinks manufacturing. In addition, transformation of each glycosidic precursor to damascenone under strong acidic conditions (pH 2 .0 and pH 3.0) were studied. Ten μg of each glycosidic precursor was incubated in aqueous hydrochloric acid with pH 2.0 (or pH 3.0) at 90°C for 10, 20, and 30 min, respectively. After

cooling to room temperature, the reaction solutions were neutralized with 0.1 M NaOH, and then 0.5 μg of phenol was added as an internal standard. Subsequently, the solutions were extracted with an azeotropic mixture of pentane- dichloromethane (2:1 v/v) three times. The resulting organic fractions were dried over anhydrous $MgSO_4$, concentrated, and then subjected to gas chromatography- mass spectrometry (GC-MS) for determination of damascenone. The residual aqueous fractions were lyophilized, dissolved in 40 μL of distilled water, and then subjected to HPLC analysis for determination of the glycosidic precursors of damascenone.

Determination of damascenone by GC-MS

Damascenone was identified and monitored using a GC 17A-MS QP5050A (Shimadzu). The GC was equipped with a capillary TC-WAX column (GL Sciences Inc., Japan), 30 m × 0.25 mm I.D., and 0.25 μm film thickness. Helium was used as a carrier gas with a flow rate of 1.6 mL/min. The samples were subjected to the injector (held at 230°C) in split mode (split ratio, 28:1). The GC oven was maintained at 130°C for 2 min. The temperature of the oven was programmed at 24°C/min to 154°C and then at 4°C/min to 230°C. Mass spectra were generated at 70 eV with 0.2 scans/sec. The mass spectrometer was operated in full scan mode (for qualitative analysis of damascenone) or in selected ion monitoring (SIM) mode for quantitative analysis. In the latter case, the mass spectrometer was focused on *m/z* 190, 121, and 69, the dominating fragments resulting from the fragmentation of damascenone.

Analysis of the glycosidic precursors of damascenone by HPLC

HPLC analysis of the glycosidic precursors were performed using a JASCO system (Japan Spectroscopic Co. Ltd., Japan) equipped with a 4.6 mm × 150 mm, 5 μm particle size, J'sphere ODS H-80 column. The sample solutions were analyzed using gradient elution with water and acetonitrile, at a flow rate of 1 mL/min at 40°C. The elution was performed using a linear

gradient from 5% to 25% acetonitrile within 20 min. UV-vis spectra were recorded between 200 and 600 nm for each chromatographic peak. The glycosidic precursors were identified based on retention time and co-injection with the authentic compounds.

Statistical analysis

Data are expressed as mean ± standard deviation. Student's t-test was used to estimate significance for comparisons. A probability level of 5% ($p < 0.05$) was considered significant.

Results and discussion

Occurrences of glycosidic precursors of damascenone in green tea infusions

It is generally accepted that many volatile compounds are stored as glycosylated precursors in plants. From a chemical point of view, glycosylated volatile compounds enhance water solubility and decrease reactivity (**Winterhalter & Skouroumounis, 1997**) compared with their free aglycone counterparts. It was proposed that the precursors of damascenone in the black tea infusions have a close relation to the aglycons released from the glycosides during the manufacturing of black tea leaves (**Kumazawa, & Masuda, 2001**). In the present study, (3*S*, 9*R*)-**1a** (89 μg), (3*R*, 9*R*)-**2a** (16 μg), and (3*R*, 9*R*)-**2b** (0.45 μg) were isolated and identified in green tea infusions (100 g of water extract) based on the evidences of HPLC-MS and NMR (**Figure 2 and Table 1**). These results prove that glycosidic precursors also naturally occur in tea leaves. Interestingly, all the glycosidic precursors of damascenone isolated from the tea leaves show the *R*-configuration at the C-9 position of the aglycone parts, whereas the 9*S*-glycosidic precursors were not detected in this study. It would be of interest to determine whether it is due to the occurrence of 9*S*-grasshopper ketone derived from neoxanthin, or chiral selectivity of glycosyltransferases responsible for the formation of the glycosidic precursors of

183 damascenone in tea leaves.

184 *Formation of damascenone from their glycosidic precursors during heating processing of green*
185 *tea infusions*

186 Glycosidic precursors of damascenone are present in nature, however they can not be
187 converted to the free volatile damascenone under natural environmental conditions. A
188 significant enhancement in the content of damascenone occurs when fresh natural products are
189 subjected to thermal-processing. For example, damascenone was neither produced nor released
190 from the flowers of *Rosa damascena* Mill., whereas it could be demonstrated that the essential
191 oil content was due to the hydrolysis of glycosidically precursors during steam distillation
192 (**Suzuki et al., 2002**). During the production of canned and bottled drinks of green and black
193 teas, a significant change in the flavor was observed based on aroma extract dilution analysis
194 (AEDA). Damascenone exhibited the highest odor potencies after heat processing and is
195 mainly responsible for the sweet flavor of heated black tea (**Kumazawa, & Masuda, 2001**). In
196 this work, damascenone was detected in the green tea infusions under similar conditions (pH 5.4
197 and 120°C for 10 min) that were observed during the production process of bottled and canned
198 green tea drinks (**Figure 3**). Interestingly, without addition of green tea infusions, the four
199 glycosidic precursors were not transformed to damascenone under equal conditions (pH 5.4 and
200 120°C for 10 min). However, enhancements in damascenone contents were observed after
201 addition of the glycosidic precursors (**1a**, **1b**, **2a**, and **2b**) to the green tea infusions (**Figure 3**).
202 This suggests that rising damascenone contents during manufacturing process may result from
203 hydrolysis or transformation of the glycoconjugate precursors at high temperatures or likely of so
204 far unidentified interactions with other metabolites. Also, it demonstrates that damascenone
205 liberated as off-flavor during manufacturing of bottled and canned green tea beverages (**Masuda,**

206 **2008**) might be originated from the hydrolysis of the glycoconjugate precursors. It will be very
207 interesting to identify components of green tea infusions which possibly affect the hydrolysis of
208 the glycosidic precursors to damascenone.
209 *Proposed pathways for the transformation of glycosidic precursors to damascenone under strong*
210 *acidic conditions*
211 To further clarify pathways of transformation of the glycosidic precursors to damascenone, we
212 used a model system with strong acidic conditions (pH 2.0 and pH 3.0). Damascenone could
213 not be released from each glycosidic precursor under slightly acidic conditions (pH 5.4) in this
214 study as described previously, although such conditions are optimal for the chemical
215 transformation of neoxanthin or megastigma-6,7-dien-3,5,9-triol into damascenone (**Bezman et**
216 **al., 2005; Isoe, Katsumura, & Sakan, 1973**). In comparison with the liberation at pH 3.0,
217 more damascenone was formed from the transformation of **1a (Figure 4A)** and **2a (Figure 4B)**
218 at pH 2.0 at 90°C for 10 to 30 min. It shows that the lower pH value of pH 2.0 favored
219 acid-catalyzed conversions of glycoconjugate precursors to damascenone. In addition, a
220 stability analysis of damascenone showed that there were no significant changes during
221 incubation of damascenone at pH 2.0 at 90°C for 10 to 30 min. By model systems it has been
222 demonstrated that pH values and reaction temperatures affect the release of volatiles from their
223 glycosides. Strong acidic conditions and high temperature increased the release from glycosidic
224 precursors, however long reaction periods increased the amount of transformation products
225 (**Skouroumounis, & Sefton, 2000**). In our model system (pH 2.0, 90°C), more damascenone
226 was formed from **1a** and **1b** during 30 min in comparison with **2a** and **2b** ($p < 0.05$, **Figure 4C**).
227 However, there are no significant different amounts of liberated damascenone between
228 hydrolysis of the C-9- and C-3-*O*-β-D-glucopyranosides. In addition, damascenone formation

(except hydrolysis of **1a)** almost increased reaction time-dependently.

Isoe et al. (**Isoe, Katsumura, & Sakan, 1973**) proposed a pathway for the biogeneration of damascenone derived from the degradation of neoxanthin with grasshopper ketone as an intermediate. Recently, it was confirmed that the carotenoid cleavage dioxygenase 1 from *Rosa damascena* is involved in the cleavage from neoxanthin to produce the grasshopper ketone (**Huang et al., 2009**). Also a formation directly from neoxanthin by acidic oxidation and thermal degradation without the involvement of enzymatic activity was demonstrated (**Bezman et al., 2005**). The two pathways suggest that megastigma-6,7-dien-3,5,9-triol (**1**) is a key intermediate in the formation of β-damasceone. Another important polyol, 3-hydroxy-7,8-didehydro-β-ionol (**2**), also has been determined as an important precursor of damascenone in wines (**Skouroumounis, Massy-Westropp, Sefton, & Williams, 1992**), and black teas (**Kumazawa, & Masuda, 2001**). It was suggested by **Puglisi et al.** (**2001**) that **1** was quite labile to form **2** at room temperature and at pH 3.0 in an aqueous environment. β-D-Glucosyl moieties at the C-3 and / or C-9 stabilize **1 (Skouroumounis, & Sefton, 2000),** but the glyconconjugates of **1a** and **1b** were dehydrated to form the glyconconjugates of **2a** and **2b** at low pH value and high temperature (pH 2.0 and 90°C) (**Figure 5**). It was also confirmed that **2a** was converted from **1a** during steam distillation of flowers of *R. damascena* Mill (**Suzuki et al., 2002**). In the present study, the transformation rates (%) from **1a** to **2a** and from **1b** to **2b** were 18% and 49%, respectively. This demonstrates that the β-D-glucosyl moiety at C-3 position results in higher dehydration rates from **1** to **2** than the transformation of **1** with sugar rediues at C-9 position. As suggested by **Skouroumounis et al. (1992)**, megastigma-4,6,7-triene-3,9-diol (**3**) and megastigma-3,5-dien-7-yn-9-ol (**4**) were identified as two intermediates in the transformation of **1** to damascenone. The glyconconjugates of **3** (**3a**

and **3b**), and **4** (**4a**) or the aglycon **4** were hypothetically proposed as intermediates involved the pathways leading from the glyconconjugates of **1** (**1a** and **1b**) to damascenone (**Figure 6**). The releases of damascenone from the glycoconjugates of **1** were higher than these from glycoconjugates of **2**, and the position of glycosylation (C-3 or C-9) did not affect significantly the transformation rates (**Figure 4C**). Therefore, we propose that **1a** (or **1b**) was more easily transformed to damascenone *via* **3a** and **4a** (or **3b** and **4**) as shown in **Figure 6**.

Conclusion

Since the original suggestion by Kumazawa and Masuda (**2001**), this study provides the evidence that the glycosidic precursors of damascenone naturally occur in tea leaves for the first time. We also confirm that an enhancement in damascenone contents after heating processes of green tea infusions is due to hydrolysis of the glycoconjugate precursors at the elevated temperatures during manufacturing. In addition, the pathways of liberation of the glycosidic precursors to damascenone under acidic conditions were characterized in the present study, which may help us to advance the understanding of formation pathways of flavor compounds during manufacturing processes of certain food products from various plant sources.

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Table 1 ^{1}H NMR data of authentic (3*S*, 9*R*)-**1a** and Fraction II-C from the green tea infusions (dissolved in CD_3OD, 500 MHz)

Authentic (3*S*, 9*R*)-1a				**Fraction II-C from the green tea**	
^{1}H	**ppm**	**mult**	***J* (Hz)**	**^{1}H**	**ppm**
2ax	1.29	dd	11.5, 9.2	**2ax**	1.30
2eq	1.89	ddd	2.3, 4.2, 11.5	**2eq**	1.91
3	4.20	m		**3**	4.20
4ax	1.42	dd	11.9, 8.8	**4ax**	1.43
4eq	2.15	ddd	2.3, 4.2, 13.2	**4eq**	2.21
8	5.45	d	6.7	**8**	5.46
9	4.48	m		**9**	4.48
10	1.32	d	6.1	**10**	1.33
11	1.10	s		**11**	1.11
12	1.24	s		**12**	1.25
13	1.36	s		**13**	1.37
1'	4.56	d	7.9	**1'**	4.56
2'	3.23	t	8.2	**2'**	3.24
3'	3.39	t	7.5	**3'**	3.39

4'	3.37	dd	7.5, 7.9	**4'**	-*
5'	3.33	ddd	1.2, 4.6, 7.9	**5'**	-*
6'a	3.71	dd	4.6, 12.2	**6'a**	3.72
6'b	3.88	dd	1.2, 12.2	**6'b**	3.89

***** The peaks were blended with peak from H_2O and not separated clearly.

Table 1 Tomomi KINOSHITA

Legends to figures:

Figure 1 A brief flow chart of isolation and purification of the glycosidic precursors of damascenone from green tea infusions.

For details refer to “Materials and methods”.

Figure 2 Mass chromatograms of authentic (3*R*, 9*R*)-**2a**, (3*R*, 9*S*)-**2a** (upper chromatogram in **A**), (3*R*, 9*R*)-**2b**, (3*R*, 9*S*)-**2b** (upper chromatogram in **B**) and selected ion traces from the HPLC-ESI/MS+ analysis at *m/z* 393 ($[M+Na]^+$), and *m/z* 409 ($[M+K]^+$) of Fraction II-A (lower chromatogram in **A**) and Fraction II-B (lower chromatogram in **B**) from the green tea infusions, and chromatograms from the HPLC chiral analysis of authentic (3*R*, 9*R*)-**2b**, (3*R*, 9*S*)-**2b** (upper chromatogram in **C**), and Fraction II-B from the green tea infusions (lower chromatogram in **C**)

Figure 3 Formations of damascenone from the glycosidic precursors (**1a, 1b, 2a, and 2b**) in the presence of green tea infusions under a similar conditions (pH 5.4 and 120°C for 10 min)

which occur during the manufacturing process of bottled and canned green tea drinks.

Ten μg of each glycosidic precursor and 1 g of boiled water extract of tea leaves were incubated at pH 5.4, 120°C, and for 10 min.

Figure 4 Effects of different pH values (2.0 and 3.0) on transformation rates of **1a (A)** and **2a (B)** to damascenone at 90 °C for 10 to 30 min, and comparison of transformation rates of glycosidic precursors (**1a, 1b, 2a, and 2b**) to damascenone at 90 °C at pH 2.0 for 10 to 30 min (**C**).

The transformation rates (%) were calculated as [amount of formed damascenone (mol)/ amount of glycosidic precursor (mol)] × 100. Data are expressed as mean ± S.D. (n=3-9). (A) **, $p < 0.01$. (B) N.D., not detected. (C) Small letters indicate significant differences ($p <$

0.05 or $p < 0.01$) between same glycosidic precursor at different reaction time (10, 20, and 30 min). Capital letters indicate significant differences ($p < 0.05$ or $p < 0.01$) between different glycosidic precursors at same reaction time.

Figure 5 Chromatograms of the residual aqueous fractions from hydrolysis of **1a (A)** and **1b (B)** at 90°C at pH 2.0 for 30 min.

After the hydrolysis liberated volatiles were extracted with pentane- dichloromethane (2:1 v/v) and the remaining aqueous layers were lyophilized. The residual aqueous fractions were analyzed by HPLC (detailed conditions are described under "Materials and methods").

Figure 6 Hypothetical pathways for the formation of damascenone from **1a** (**A**) and **1b** (**B**) at

90°C at pH 2.0 for 30 min.

Compound **1a,** 9-*O*-β-D-glucopyranosyl-megastigma -6,7-dien-3,5,9-triol; **1b,** 3-*O*-β-D-glucopyranosyl-megastigma -6,7-dien-3,5,9-triol; **2a,** 9-*O*-β-D-glucopyranosyl-3-hydroxy-7,8-didehydro-β-ionol; **2b**, 3-*O*-β-D-glucopyranosyl-3- hydroxy-7,8-didehydro-β-ionol; **3a,** 9-*O*-β-D-glucopyranosyl- megastigma-4,6,7-triene-3,9-diol; **3b,** 3-*O*-β-D-glucopyranosyl-megastigma-4,6,7-triene-3,9-diol; **4**, megastigma-3,5-dien-7-yn-9-ol; **4a**, 9-*O*-β-D-glucopyranosyl-megastigma-3,5-dien-7-yn-9-ol. * The transformation rates (%) from compound **1a** or **1b**. The bold arrows indicate the pathways with comparatively high flux, whereas light arrows indicate those with lower flux. The compounds shown in the dashed rectangle are the proposed intermediates.

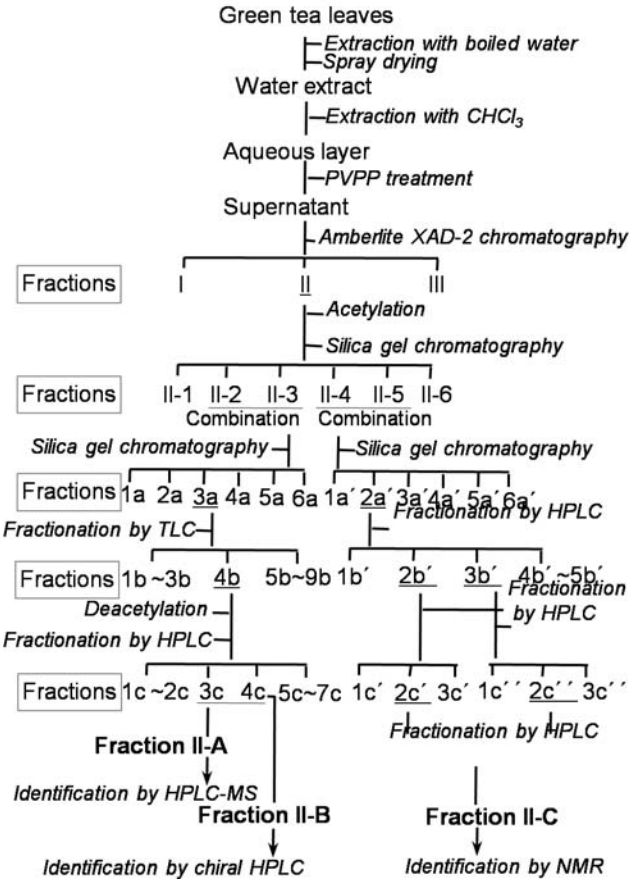
Green tea leaves
Extraction with boiled water
Spray drying
Water extract
Extraction with CHCl3
Aqueous layer
PVPP treatment
Supernatant
Amberlite XAD-2 chromatography
Fractions
I II III
Acetylation
Silica gel chromatography
Fractions
II-1 II-2 II-3 II-4 II-5 II-6
Combination Combination
Silica gel chromatography
Silica gel chromatography
Fractions
1a 2a 3a 4a 5a 6a 1a´ 2a´ 3a´ 4a´ 5a´ 6a´
Fractionation by HPLC
Fractionation by TLC
Fractions
1b~3b 4b 5b~9b 1b´ 2b´ 3b´ 4b´~5b´
Fractionation by HPLC
Deacetylation
Fractionation by HPLC
Fractions
1c~2c 3c 4c 5c~7c 1c´ 2c´ 3c´ 1c´´ 2c´´ 3c´´
Fractionation by HPLC
Fraction II-A
Identification by HPLC-MS
Fraction II-B
Fraction II-C
Identification by chiral HPLC
Identification by NMR

Figure 1 Tomomi KINOSHITA

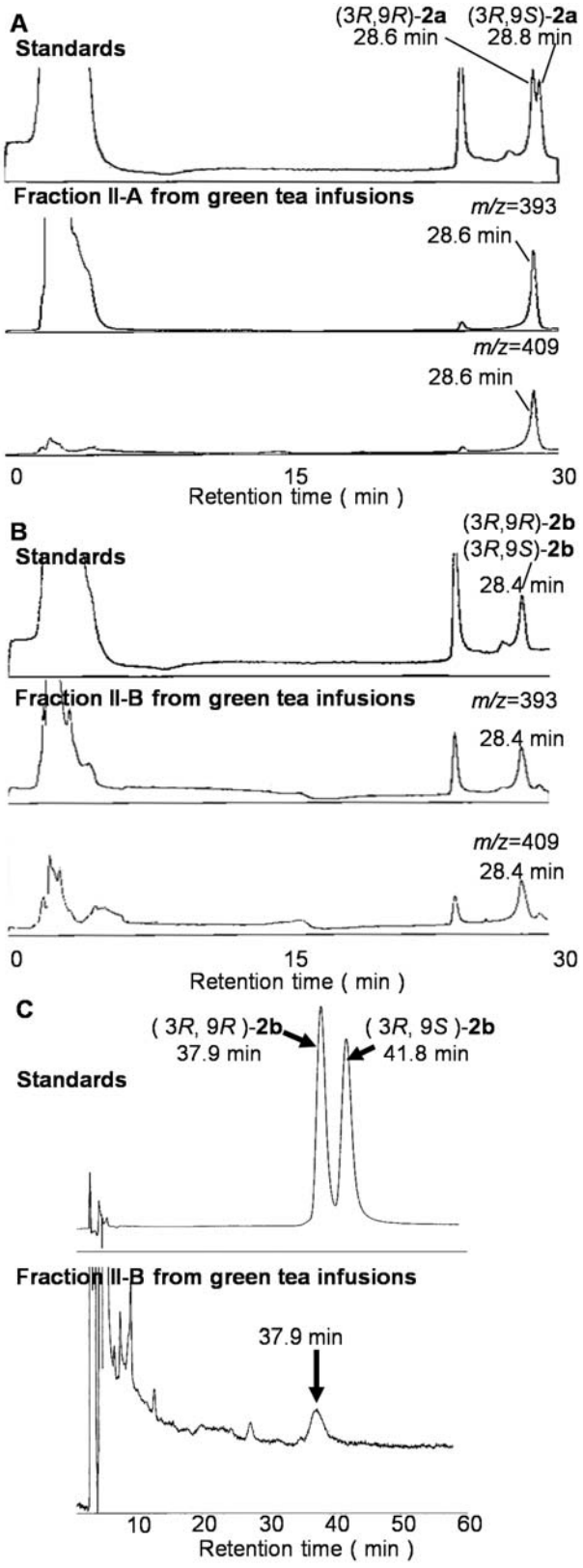
A
Standards
(3R,9R)-2a
28.6 min
(3R,9S)-2a
28.8 min
Fraction II-A from green tea infusions
m/z=393
28.6 min
m/z=409
28.6 min
0
15
30
Retention time (min)
B
Standards
(3R,9R)-2b
(3R,9S)-2b
28.4 min
Fraction II-B from green tea infusions
m/z=393
28.4 min
m/z=409
28.4 min
0
15
30
Retention time (min)
C
(3R, 9R)-2b
37.9 min
(3R, 9S)-2b
41.8 min
Standards
Fraction II-B from green tea infusions
37.9 min
10
20
30
40
50
60
Retention time (min)

Figure 2 Tomomi KINOSHITA

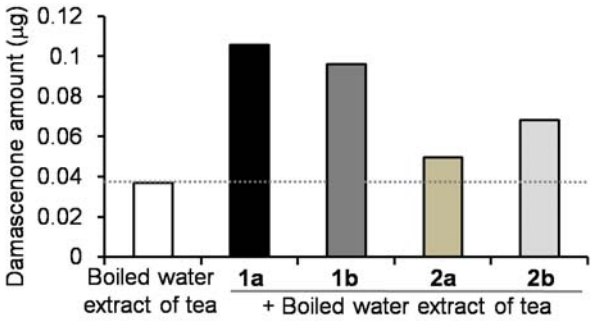

Figure 3 Tomomi KINOSHITA

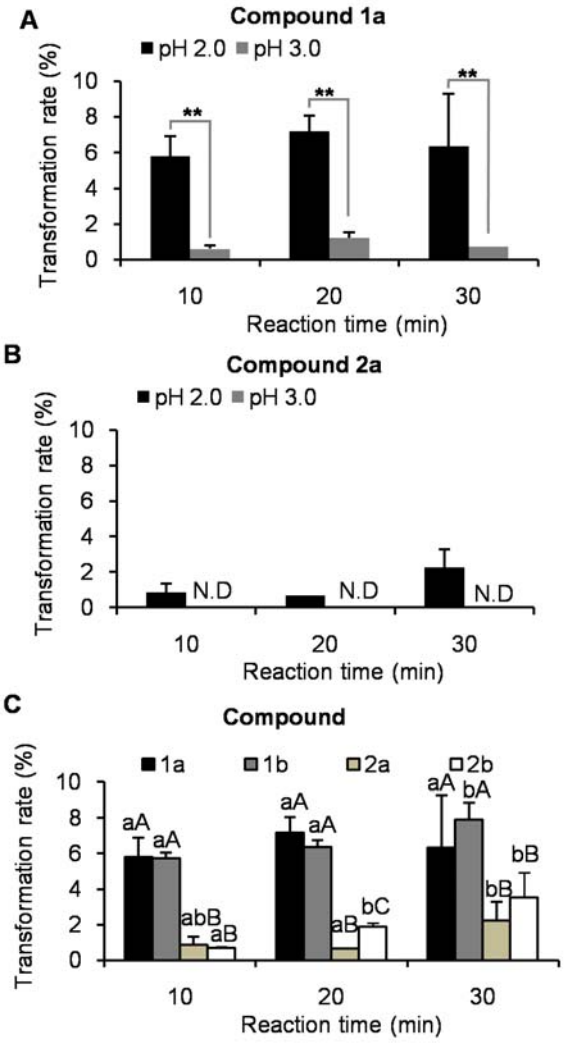
A
Compound 1a
pH 2.0
pH 3.0
Transformation rate (%)
10
8
6
4
2
0
**
**
**
10
20
30
Reaction time (min)
B
Compound 2a
pH 2.0
pH 3.0
Transformation rate (%)
N.D
N.D
N.D
10
20
30
Reaction time (min)
C
Compound
1a
1b
2a
2b
Transformation rate (%)
aA
aA
abB
aB
aA
aA
aB
bC
aA
bA
bB
bB
10
20
30
Reaction time (min)

Figure 4 Tomomi KINOSHITA

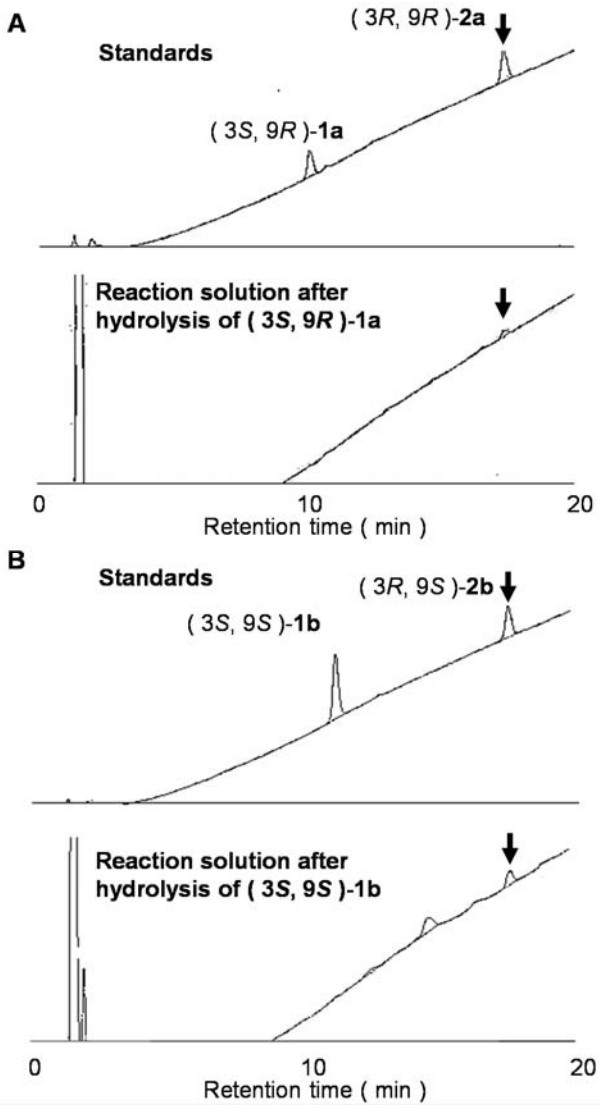

Figure 5 Tomomi KINOSHITA

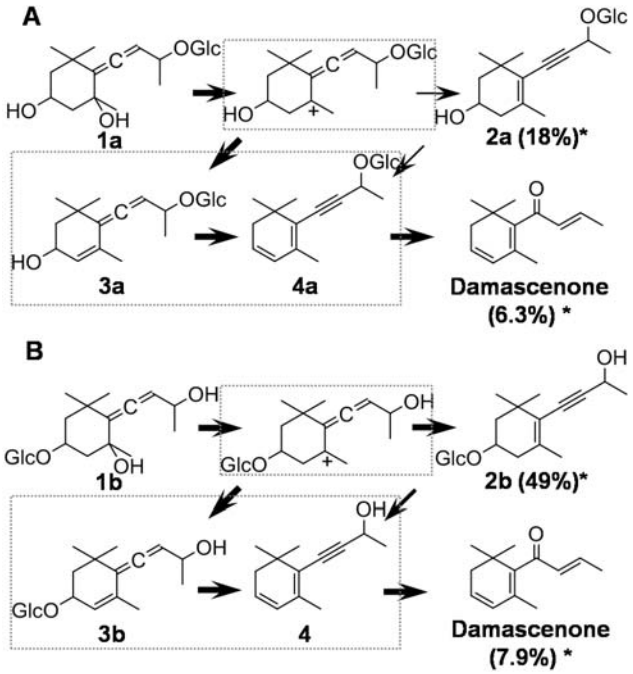
A
OGlc
HO
OH
1a
2a (18%)*
3a
4a
Damascenone
(6.3%) *
B
GlcO
1b
2b (49%)*
3b
4
Damascenone
(7.9%) *

Figure 6 Tomomi KINOSHITA