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Emission of 2-phenylethanol from its β-D-glucopyranoside and the biogenesis of these compounds from [$^2H_8$] L-phenylalanine in rose flowers

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Abstract—The hydrolysis of 2-phenylethyl β-D-glucopyranoside (**3**) was found to be partially inhibited by feeding with 2-phenyl-*N*-glucosyl-acetamidiumbromide (**8**), a β-glucosidase inhibitor, resulting in a decrease in the diurnal emission of 2-phenylethanol (**2**) from *Rosa damascena* Mill. flowers. Detection of [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**2** and [1,2,2′,3′,4′,5′,6′-$^2H_7$]-**2** from *R.* 'Hoh-Jun' flowers fed with [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**3** suggested that β-glucosidase, alcohol dehydrogenase, and reductase might be involved in scent emission. Comprehensive GC-SIM analyses revealed that [1,2,2,2′,3′,4′,5′,6′-$^2H_8$]-**2** and [1,2,2,2′,3′,4′,5′,6′-$^2H_8$]-**3** must be biosynthesized from [1,2,2,2′,3′,4′,5′6′-$^2H_8$] L-phenylalanine ([$^2H_8$]-**1**) with a retention of the deuterium atom at α-position of [$^2H_8$]-**1**.

1. Introduction

Floral scent compounds, such as monoterpenoids, shikimate derived compounds, and fatty acids-derived compounds, are synthesized in some flowers. These compounds are emitted to attract pollinators,[1−7] to attract or to deter herbivores,[8] and/or to prevent bacterial and fungal infections.

2-Phenylethanol (**2**) is one of the dominant floral scent compounds emitted from roses such as *Rosa damascena* spp and *R.* 'Hoh-Jun'. Rhythmic cycles in the emission of **2** are observed in *R.* 'Hoh-Jun' as shown in Figure 2, although *R. damascena* Mill. transiently emits **2** (cf. Fig. 5). It has been proposed that glycosidically bound volatiles act as aroma precursors,[9−12] and their hydrolysis may be responsible for the rhythmic emission pattern observed in *R.* 'Hoh-Jun'.[13] In *R. damascena* Mill. flowers, the amount of 2-phenylethyl β-D-glucoside (**3**) is greater during the stages of early development and decreases during the unfurling process, whilst levels of the free form generally increased over the unfurling.[14] In *Narcissus* flowers, β-glucosidase activity was found to be involved in the scent emission detected by gas sensors.[15] To date, attempts to correlate concentrations of glucosides with rhythmic cycles in volatile emission have been unsuccessful.[16,17] Synchronous diurnal rhythms have been detected in the concentration of both **2** and its acetate, but not in the concentration of **3**, in the *Trifolium repens* flowers.[17]

Although there has been much progress in elucidating biosynthetic pathways and characterizing enzymes, the underlying biosynthetic route of **2** in flowers has yet to be fully understood. We recently reported the biogenesis of **2** from L-phenylalanine (**1**) in rose flowers.[18] In that study, [1,2,2,2′,3′,4′,5′,6′-$^2H_8$]-**1** ([$^2H_8$]-**1**) was fed to *R.* 'Hoh-Jun' and *R. damascena* Mill. flowers throughout maturation, and feeding was terminated at full bloom stage. On the basis of GC-MS analyses, we found that [$^2H_8$]-**1** was incorporated into both **2** and **3** when the flowers were fed until full bloom. In both rose species, the labeling pattern of **2** was almost identical to that of **3**, and indicated the presence of both [$^2H_7$]- and [$^2H_8$]-**2**, and [$^2H_7$]- and [$^2H_8$]-**3**. This may be ascribed to the equilibrium established between **2** and **3**. Although the labeling pattern for **2** and **3** established that these compounds were produced from **1** via several routes as

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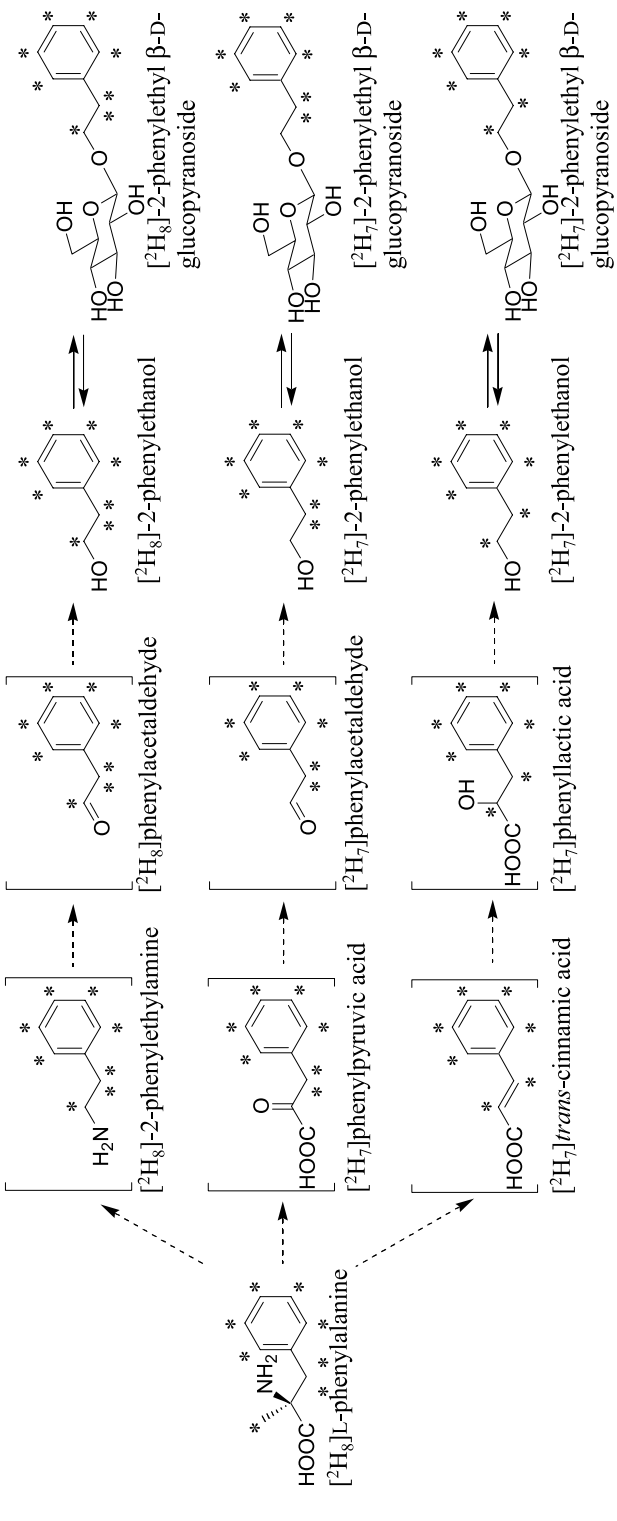

Figure 1. Hypothetical biogenetic pathway for 2-phenylethanol (**2**) and 2-phenylethyl β-D-glucopyranoside (**3**) from L-phenylalanine (**1**). Asterisk denotes the position of a ^{2}H atom.

shown in Figure 1, the route involving phenylpyruvic acid (**4**) was thought to be the major one. However, the detection of [$^2H_8$]-**2** and [$^2H_8$]-**3** strongly suggested the presence of a biosynthetic route involving the intermediate 2-phenylethylamine (**6**).

In this paper, we describe the involvement of β-glucosidase in the emission of **2**, based on experiments using a β-glucosidase inhibitor and feeding with [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**3**, as well as details of the biosynthetic pathways of **2**, deduced from feeding with [$^2H_8$]-**1**, in *R. damascena* Mill. and *R.* 'Hoh-Jun' flowers.

2. Results and discussion

In *R. damascena* Mill. flowers, the amount of **3** is greater in early development and declines over the unfurling process, whereas levels of the free form generally increased over the unfurling process under natural conditions.[14] As shown in Figures 2 and 5, we found that **2** was emitted in higher concentrations when exposed to light than when kept in the dark at a constant temperature. Loughrin[16] has suggested that changes in the rate of the glycoside hydrolysis may be responsible for the emission of scent compounds. We therefore attempted to verify the involvement of β-glucosidase and its substrate **3** in the production of **2** in *R. damascena* Mill. flowers.

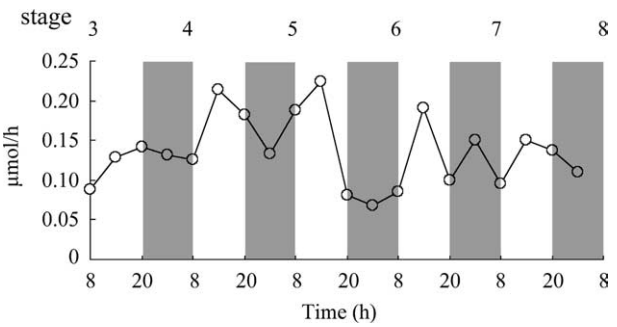

Figure 2. 2-Phenylethanol (**2**) emission during the unfurling process in *Rosa* 'Hoh-Jun' flowers of intact plants maintained under a 12-h photo period at constant temperature (22 °C) and relative humidity (75%). Shaded and non-shaded areas correspond to exposure to dark and light, respectively. Stages of flowers are defined in the text.

We examined the effect of 2-phenyl-*N*-β-glucosyl-acetamidiniumbromide (**8**, Fig. 3), a glucosidase inhibitor,[19,20] on the hydrolytic activity of crude enzymes prepared from flower petals. The crude enzymes (prepared from 100 to 400 mg of flower petals) released 1.3 μmol of **2** in 1 h from 5 μmol of **3**, and more than 95% of the hydrolytic activity was inhibited by 0.6 μmol of **8**. As each flower accumulated 15 μmol of **3** at stage 3, it was expected that more than 2.0 μmol of **8** would inhibit the hydrolysis of **3** in each flower. Therefore, 300 μl of an aqueous solution of **8** (10 mM) was fed to *R. damascena* Mill. flowers at stage 3. As shown in Figure 4b, the level of **3** gradually decreased by 7 μmol/flower in the control group, whereas the level in the fed group was almost constant (16–14 μmol/flower) during the dark period (6–18 h after the feeding commenced). During the day-time (18–30 h after the feeding commenced) the level of **3** remained fairly constant (13–17 μmol/flower) in the fed group, but decreased by 5 μmol/flower in the control group. The concentration of **2** in the tissue increased gradually to reach its maximal level (17 μmol/flower) 24 h after the feeding commenced in the control group, but the concentration did not exceed 5 μmol/flower during the unfurling process in the fed group (Fig. 4a).

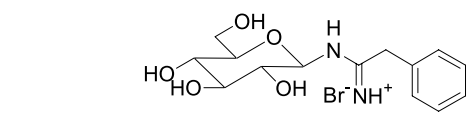

Figure 3. 2-Phenyl-*N*-β-glucopyranosyl-acetamidiumbromide (**8**), a β-glucosidase inhibitor.

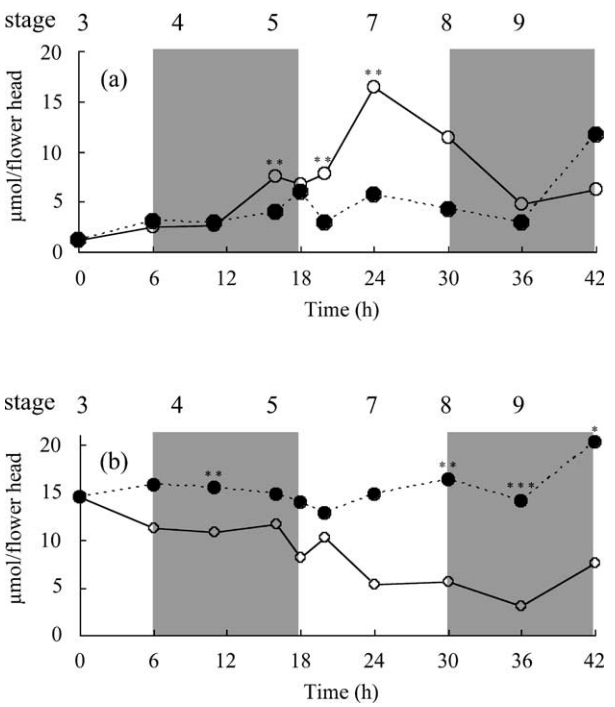

Figure 4. Effect of 2-phenyl-*N*-β-glucopyranosyl-acetamidiumbromide (**8**) on the accumulation of 2-phenylethanol (**2**) (a) and 2-phenylethyl β-D-glucopyranoside (**3**) (b) accumulated in the flowers of *Rosa damascena* Mill. during the unfurling process. ○, Control group; ●, fed **8**. Intact plants were exposed to the same conditions as in Figure 2. Shaded and non-shaded areas correspond to exposure to dark and light, respectively. $^{***}P<0.01$, $^{**}P<0.05$, $^{*}P<0.1$ for paired comparisons of the control versus the fed group. Stages of flowers are defined in the text.

As shown in Figure 5, diurnal emission reached its maximum level at 21 h after feeding commenced in the control group, whereas a peak was not observed in the fed group. β-Glucosidase activity increased between stages 4 and 6 (Fig. 5). The increase in β-glucosidase activity was observed prior to the increase in the concentration of **2** in the control flowers. As **8** does not bind to β-glucosidase via a covalent bond,[20] similar level of β-glucosidase activity was detected in the crude extract even in the fed group. Although the high β-glucosidase activity at stage 9 cannot be rationally explained, these observations strongly suggest that β-glucosidase activity is involved in both the production and the emission of **2**. We therefore, focus our research on the effect of the inhibitor, **8**, on the rhythmic emission of **2** observed in *R.* 'Hoh-Jun' and *R. damascena* semperflorens quatra saisons[21] flowers

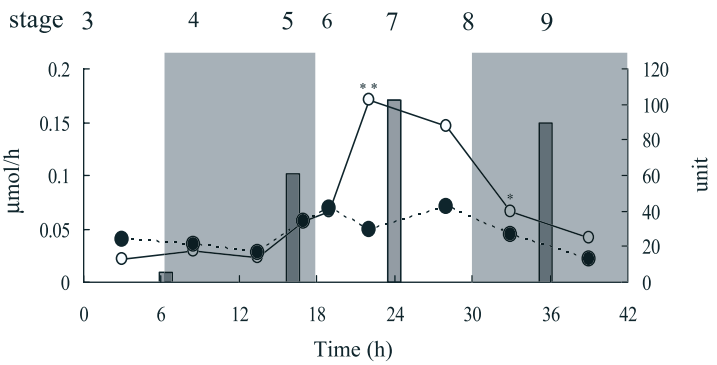

Figure 5. Effect of 2-phenyl-*N*-β-glucopyranosyl-acetamidiumbromeide (**8**) on 2-phenylethanol (**2**) emission from *Rosa damascena* Mill. flowers, and changes in β-glucosidase activity during the unfurling process *R. damascena* Mill. flowers. ○, Control group; ●, fed **8**. Bar indicates β-glucosidase activity. Intact plants were exposed to the same conditions as in Figure 2. Shaded and non-shaded areas correspond to exposure to dark and light, respectively. $^{**}P<0.05$, $^{*}P<0.1$ for paired comparisons of the control versus the fed group. Stages of flowers are defined in the text.

During the study, [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**3** was fed to *R.* 'Hoh-Jun' flowers at stage 3 to confirm the emission of [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**2**. As shown in Figure 6, [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**2** and [1,2,2′,3′,4′,5′,6′-$^2H_7$]-**2** were detected in sample of the head-space gas, and the ratio of the [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**2** to [1,2,2′,3′,4′,5′,6′-$^2H_7$]-**2** decreased during the unfurling process until senescence stage (between stages 3 and 8). This observation strongly suggests that [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**2**, once produced by endogenous β-glucosidase, is transformed to [1,2,2′,3′,4′,5′,6′-$^2H_7$]-phenylacetaldehyde (**5**) by alcohol dehydrogenase, and subsequently interconverted into [1,2,2′,3′,4′,5′,6′-$^2H_7$]-**2**. In our preliminary experiments, we detected alcohol dehydrogenase activities toward **2** in the crude enzymes prepared from *R.* 'Hoh-Jun' petal tissues (data not shown). This indicates that the oxidation–reduction process is a significant factor involved in regulating the production and emission of **2**.

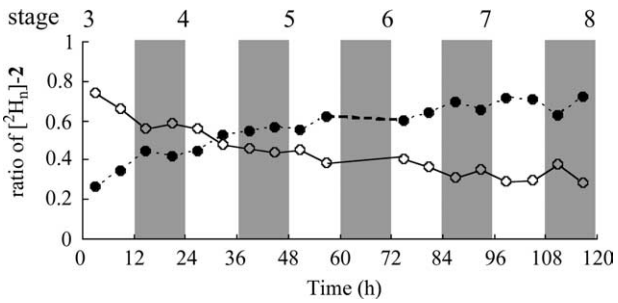

Figure 6. Changes in the ratio of [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**2** to [1,2,2′,3′,4′,5′,6′-$^2H_7$]-**2** emitted from the flowers of *Rosa* 'Hoh-Jun' after feeding with [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**3**. ○, [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**2**; ●, [1,2,2′,3′,4′,5′,6′-$^2H_7$]-**2**. Intact plants were exposed to the same conditions as in Figure 2. Stages of flowers are defined in the text.

We recently found that [$^2H_8$]-**1** is transformed to **2** and its β-D-glucopyranoside **3** via **4**, and we proposed that this is the main biosynthetic pathway of **2** and **3** in *R. damascena* Mill. and *R.* 'Hoh-Jun' flowers.[18] The proposed pathway and the labeling pattern are illustrated in Figure 1;[18] this pathway has been already observed in yeasts and various plant tissues.[22–24] In our experiments, not only [$^2H_7$]-**2** and [$^2H_7$]-**3**, the primary isotopomers of the deuterium labeled compounds, but also [$^2H_8$]-**2** and [$^2H_8$]-**3** were detected as minor components in the flowers. This suggests that the flowers can produce **2** and **3** from **1** via several pathways from **1**. As shown in Figure 6, one deuterium atom of [1,2,2′,3′,4′,5′,6′-$^2H_8$]-**2** was lost during the oxidation–reduction reaction yielding [2,2′,3′,4′,5′,6′-$^2H_7$]-**2**. This prompted us to reconsider the biosynthetic route from **1** to **2** during feeding with [$^2H_8$]-**1**, because [$^2H_7$]-**2** and [$^2H_7$]-**3** are likely to be derived from [$^2H_8$]-**2** and [$^2H_8$]-**3**, respectively. Below, we examined the effects of [$^2H_8$]-**1** feeding over a short unfurling process in order to elucidate the actual biosynthetic route of **2** by tracing changes in the labeling patterns of [^2H_n]-**2** and [^2H_n]-**3**.

Initially, we attempted to confirm the transformation of [$^2H_8$]-**1** to [^2H_n]-**2** and [^2H_n]-**3** by increasing the concentration of [$^2H_8$]-**1** that was fed. Compound [$^2H_8$]-**1** was fed to the flowers for 48 h after unfurling initiated (stage 3), and the flower petals were collected and extracted with pentane in a microwave oven. In the previous paper,[18] all [^2H_n]-**2** were detected at the same t_R, but in this study, each [^2H_n]-**2** isotopomers was detected at different t_Rs (18.23, 18.30, and 18.32 min for [$^2H_8$]-**2**, [$^2H_7$]-**2**, and [$^2H_6$]-**2**, respectively, due to the isotope effects under the conditions used (see Section 3). Therefore, the amount of each isotopomer of [^2H_n]-**2** was estimated from the GC-SIM traces at *m/z* 130 for [$^2H_8$]-**2**, *m/z* 129 for [$^2H_7$]-**2**, and *m/z* 128 for [$^2H_6$]-**2** at each t_R. The ratio of [^2H_n]-**2** plus [^2H_n]-**3** to **2** plus **3** increased as the concentration of [$^2H_8$]-**1** increased (measured ratio 0.00033, 0.0010, 0.0032, 0.0036, 0.0073, and 0.013 at a concentration of 2.1, 4.3, 8.7, 17.3, 34.5, and 69.0 mM, respectively). This indicated that **1** is an actual precursor of **2** in the flowers. The overall yield of [^2H_n]-**2** plus [^2H_n]-**3** was ca. 1.1% when all of the [$^2H_8$]-**1** fed was absorbed in the flowers. As [$^2H_8$]-**1** contains ca. 15% of [$^2H_7$]- and/or [$^2H_6$]-**1** on the basis of ^1H NMR analyses of the methyl ester, the total empirical peak height for [$^2H_7$]-**2** must be subtracted by 15% of each value. The amount of [$^2H_6$]-**2** was not considered because the peak (which accounted for less than 5% of the total amount of [^2H_n]-**2**) for [$^2H_6$]-**2** might have originated from [$^2H_6$]-**1** at the initial feeding stage. On the basis of the prerequisite condition, the ratio ([$^2H_8$]-/[$^2H_7$]-**2**=50–58/40–42, [$^2H_8$]-/[$^2H_7$]-**3**=50–58/40–42) remained almost

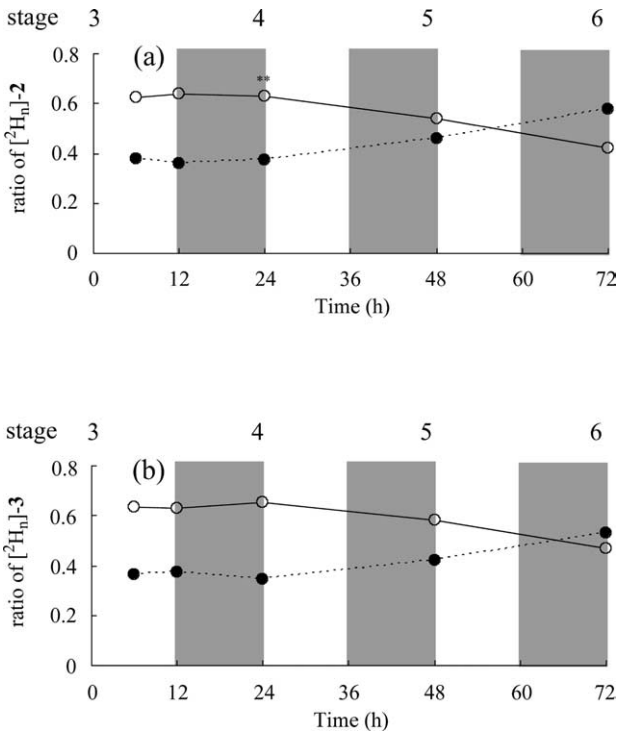

Figure 7. Changes in the ratio of [$^2H_8$]-**2** to [$^2H_7$]-**2** detected in the volatile fraction (a) and as the aglycone of **3** (b) in the flowers of *Rosa* 'Hoh-Jun' after feeding with [$^2H_8$]-**1**. ○, [$^2H_8$]-**2**; ●, [$^2H_7$]-**2**. Intact plants were exposed to the same conditions as in Figure 2.

identical, regardless of the increase in the concentration of the fed [$^2H_8$]-**1**.

We next compared changes in the $^2H_8$]-**2**/[$^2H_7$]-**2** and [$^2H_8$]-**3**/[$^2H_7$]-**3** ratios over time. Feeding of [$^2H_8$]-**1** was started at stage 3 of the *R*. 'Hoh-Jun' flower, and the flowers were subsequently collected at 6, 12, 24, 48, and 72 h after feeding began. Most of the [$^2H_8$]-**1** solution was absorbed by 72 h. The flowers were successively extracted by pentane and ethyl acetate in a microwave oven yielding volatile and glycosidic fractions as previously reported by Oka et al.[14]

As shown in Figure 7(a) and (b), changes in the ratio of [$^2H_8$]-**2**/[$^2H_7$]-**2** change corresponded to those in the ratio of [$^2H_8$]-**3**/[$^2H_7$]-**3** throughout the unfurling process (between stages 3 and 6). The ratio of [$^2H_8$]-**2**/[$^2H_7$]-**2** and [$^2H_8$]-**3**/[$^2H_7$]-**3** was 63/37, and 62/38, respectively, at the initial sampling point, but had decreased to reach 42/58 and 47/53, respectively, by 72 h after the feeding commenced. This correlation indicates that an equilibrium exists between **2** and **3** in the petal tissue, and abstracting one of the deuterium atoms from [$^2H_8$]-**2** by the action of alcohol dehydrogenase caused a decline in the ratio. The difference between the current value and the previously reported ratio [$^2H_8$]-**2**/[$^2H_7$]-**2**=[$^2H_8$]-**3**/[$^2H_7$]-**3**=10–20/90–80)[18] must be due to this abstraction of a deuterium atom from the initial isotopomer, [$^2H_8$]-**2**, during longer duration of the previous study. Therefore, **2** must be converted from **1** without the loss of a hydrogen atom at the α-position.

Interestingly, the postulated intermediates, [$^2H_8$]-**5** and [$^2H_8$]-**6**,[25] were not detected in *R*. 'Hoh-Jun' flowers upon feeding with [$^2H_8$]-**1**. This might be due to the rapid metabolism of [$^2H_8$]-**1** to [$^2H_8$]-**2** in the flower petals. Therefore, we fed 34.5 μmol of [1,1,2,2,2′,3′,4′,5′,6′-$^2H_9$]-**6**, to the *R*. 'Hon-Jun' flowers at stage 4 for 96 h to verify whether **2** and/or **3** would be produced via **6**. The flowers were extracted successively with pentane and ethyl acetate to test for [$^2H_8$]-**2** and/or [$^2H_8$]-**3**. [$^2H_8$]-**2** was not detected in the volatile fraction, although [1,2,2,2′,3′,4′,5′,6′-$^2H_8$]-**3** was detected in a trace amounts (0.0072 μmol, yield=0.021%). This yield was significantly lower than that (11%) from [$^2H_8$]-**1**, indicating that **6** might not be involved in the biosynthesis of **2** from **1**.

As previously reported[26], **5**, a key intermediate in the biosynthetic pathway, has been synthesized from **1** by a myeloperoxidase/hydrogen peroxide/chloride system derived from neutrophils. In a cell-free system, **5** is also non-enzymatically produced from **1** in the presence of the *ortho*-quinone of catechin, during which Schiff-base formation with **1** has been proposed as an intermediate.[27] Recently, a cytochrome P450 dependent oxidase,

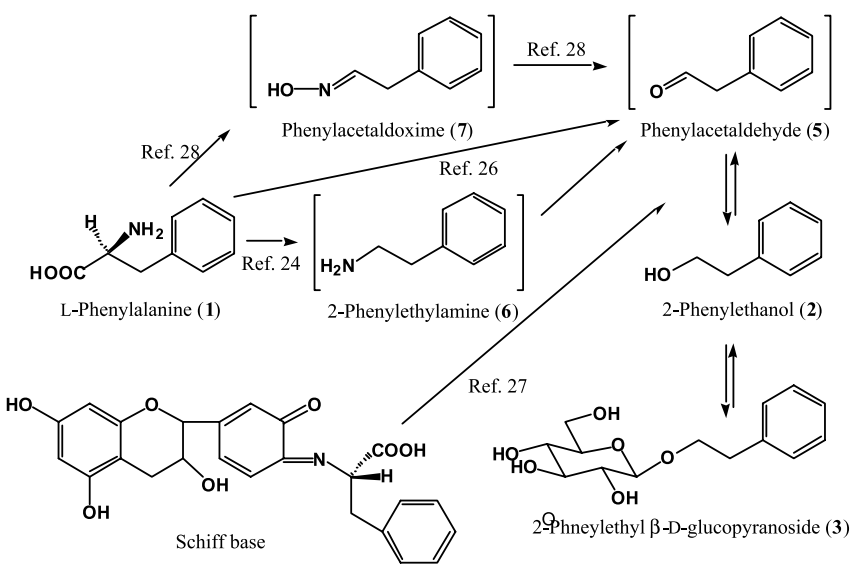

Figure 8. Possible biogenetic pathways for **2** from **1** in the rose flowers.

CYP79A2, has been characterized in *Arabidopsis* and has been shown to oxidize **1**, yielding phenylacetaldoxime (**7**).[28] Compound **7** can be enzymatically or non-enzymatically oxidized yielding **5**. In these cases, a deuterium atom at the α-position of [$^2H_8$]-**1** must be retained to yield [$^2H_8$]-**5**, which then subsequently yields [$^2H_8$]-**2**. Furthermore, **7** has been identified among volatile compounds in the flower concrete of *Michelia champaca* L.[29] We therefore, examined whether **7** is an intermediate in the biosynthetic pathway in *R.* 'Hoh-Jun' flowers. We injected [1,2,2′,3′,4′,5′,6′-$^2H_7$]-**7** (14 μmol/μl of DMSO) into *R.* 'Hoh-Jun' flowers. The overall yield (0.82 μmol, 5.8%) of [1,2,2′,3′,4′,5′,6′-$^2H_7$]-**2** (0.4%) and [1,2,2′,3′,4′,5′,6′-$^2H_7$]-**3** (5.4%) from [1,2,2′,3′,4′,5′,6′-$^2H_7$]-**7** was determined on the basis of the GC-MS analyses of the pentane and ethyl acetate fractions extracted from flowers detached 24 h after injection, suggesting that **7** is involved in the biosynthesis of **2**. Although the transformation of deuterium labeled **5** has not been confirmed yet, we can propose a plausible biosynthetic pathway for **2** as shown in Figure 8, on the basis of the results so far.

We are now focusing on the identification of the hypothesized intermediates, such as [$^2H_8$]-**5** and [$^2H_8$]-**7**, in order to clarify the exact pathway that yields [$^2H_8$]-**2** and [$^2H_8$]-**3** from [$^2H_8$]-**1** in rose petals.

In conclusion, β-glucosidase is thought to be partly involved in controlling diurnal emission of **2** in the *R. damascena* Mill. flowers. In addition, our analyses of the flower tissue after feeding have enabled us to propose a novel biosynthetic pathway for **2** and **3** via [$^2H_8$]-**5** from [$^2H_8$]-**1** in rose flowers.

3. Experimental

3.1. Chemicals and biochemicals

2,3,3,2′,3′,4′,5′,6′-[$^2H_8$] L-Phenylalanine ([$^2H_8$]-**1**, 98 atom% ^2H, Aldrich) was used. In the ^1H NMR spectral analyses of the methyl ester of [$^2H_8$]-**1**, the $^2H/H$ ratio was evaluated to be 17/83, 3/97, and 4/96 for H-2,3,3, and 2′-6′, respectively. 2-Phenyl-*N*-glucosyl-acetamidiumbromide (**8**) was synthesized by the method of Hiratake et al.[19,20] β-Glucosidase (EC 3.2.121, 500 units/mg from almond, Sigma) and naringinase (300 units/mg from *Penicillium decumbens*, Sigma) were used for enzymatic hydrolysis of glycoconjugated volatile compounds.

3.1.1. Synthesis of [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-2-phenylethyl β-D-glucopyranoside ([1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-3) [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**2** was synthesized by reduction with 9-BBN (30 mmol) in THF (10 ml) from [1,1,2,2′,3′,4′,5′,6′-$^2H_8$] styrene (2.00 g, 17.8 mmol, 98 atom% ^2H, Cambridge Isotope Laboratories, MA, USA), although the labeling pattern is not exactly the same as one of the isotopomers, [1,2,2,2′,3′,4′,5′,6′-$^2H_8$]-**2**, produced from [$^2H_8$]-**1**. The reaction mixture was treated with 6 M aq. NaOH (30 ml) and 35% H_2O_2 (30 ml). The desired compound was obtained after silica gel chromatography (hexane–EtOAc) yielding 2.38 g of a fraction containing both [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**2** and the solvent. The fraction (1.00 g, 42% of the total) was dissolved in MeCN, and stirred for 24 h at ambient temperature in the presence of 10.4 g (25.0 mmol) of 2,3,4,6-tetra-*O*-acetyl-1-bromo α-glucose (Sigma), $Hg(CN)_2$ (2.80 g, Sigma), and molecular sieves (4 Å). After purification, [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-2-phenylethyl 2,3,4,6-tetra-*O*-acetyl β-D-glucolyranoside, it was treated with 5 ml of MeONa (1 M) in MeOH–$CHCl_3$ (1:1), followed by Dowex 50W-×4 (H^+ from), and the product was purified by silica gel chromatography ($CHCl_3$–MeOH) to yield [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**3** (889 mg, 3.00 mmol, 40.1% from [1,1,2,2′,3′,4′,5′,6′-$^2H_8$] styrene). FABMS (pos.) analyses gave an ion peak at *m/z* 293 $[M+H]^+$. ^1H NMR (500 MHz, CD_3OD) δ_H: 2.93 (1H, s, H-2), 3.22 (1H, dd, *J*=8.7, 8.9 Hz, H-2″), 3.35 (1H, t, *J*=8.9 Hz, H-4″), 3.39 (1H, ddd, *J*=8.9, 5.4, 2.2 Hz, H-5″), 3.45 (1H, t, *J*=8.9 Hz, H-3″), 3.69 (1H, dd, *J*=11.7, 5.4 Hz, H-6″a), 3.99 (1H, dd, *J*=11.7, 2.2 Hz, H-6″b), 4.45 (1H, d, *J*=8.7 Hz, H-1″); ^{13}C NMR (125 MHz, CD_3OD) δ_C: 37.4 (t, *J*=19.2 Hz, C-2), 63.6 (C-6″), 72.4 (C-4″), 72.7 (quin. *J*=21.6 Hz, C-1), 75.9 (C-2″), 78.6 (C-3″), 78.6 (C-5″), 105.1 (C-1″), 128.8 (t, *J*=25.8 Hz, C-6′), 131.0 (2C, t, *J*=23.8 Hz, C-5′,7′), 131.4 (2C, t, *J*=24.8 Hz, C-4′,8′), 141.2 (C-3′). GC-MS analyses gave ion peaks at *m/z* 130 $[M]^+$ (100%) and *m/z* 129 $[M-1]^+$ (19%) for [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**2**, and peaks at *m/z* 122 $[M]^+$ (100%) and *m/z* 123 $[M+1]^+$ (8.3%) for **2**, respectively. On the basis of these data, the ratio of the [$^2H_7$]-isotopomer in [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**2** was calculated to be 17%. The ratio of [$^2H_7$]-isotopomer (17%) in [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**3** was also estimated by GC-MS of the products obtained from [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**3** after hydrolysis with β-glucosidase (from almond).

3.1.2. Synthesis of [1,1,2,2,2′,3′,4′,5′,6′-$^2H_9$]-2-phenyletylamine (6). $LiAlD_4$ (207 mg, 4.95 mmol, Aldrich) and anhydrous $AlCl_3$ (264 mg, 1.98 mmol, Sigma) were added to diethyl ether (6 ml), and stirred for 1 h under ice-cooling. [1,1,2′,3′,4′,5′,6′-$^2H_7$]-Phenylacetonitrile (204 mg, 1.65 mmol, Aldrich) was added and stirred for 1 h at ambient temperature. [1,1,2,2,2′,3′,4′,5′,6′-$^2H_9$]-**6** was obtained after treatment with 1 M NaOH and extraction with diethyl ether (197 mg, 1.50 mmol, yield 91%). EIMS analyses gave a peak at *m/z* 131 $[M]^+$; ^{13}C NMR (125 MHz, CD_3OD) δ_C: 39.1 (quin., *J*=19.1 Hz, C-2), 43.3 (quin., *J*=21.0 Hz, C-1), 126.7 (t, *J*=24.3 Hz, C-4′), 129.0 (2C, t, *J*=24.3 Hz, C-2′, 6′), 129.3 (2C, t, *J*=23.8 Hz, C-3′,5′), 140.7 (s, C-1′). The deuterium ratio was not estimated.

3.1.3. Synthesis of [1,2,2′,3′,4′,5′,6′-$^2H_7$]-phenylacetaldoxime (7). [1,2,2′,3′,4′,5′,6′-$^2H_7$]-phenylacetaldoxime (**7**) was prepared from [1,2,2′,3′,4′,5′,6′-$^2H_7$]-1-nitrostyrene[30] according to the method of Sera et al.[31] [1,2,2′,3′,4′,5′,6′-$^2H_7$]-1-Nitrostyrene (500 mg, 3.3 mmol) was treated by lead powder (5.5 g, 33 mmol) in acetic acid-DMF (1.5:20 v/v, 21.5 ml) for 2 h at ambient temperature. The reaction mixture was added to water (20 ml) and extracted with diethyl ether (20 ml×3 times) yielding crude extract, which was then purified by column chromatography on silica gel (hexane–diethyl ether) to yield [1,2,2′,3′,4′,5′,6′-$^2H_7$]-**7** (340 mg, 2.4 mmol, yield 73%). EIMS *m/z* 142 $[M]^+$; ^{13}C NMR (125 MHz, CD_3OD) δ_C: 31.1 (t, *J*=19.5 Hz, C-2 of *Z*-isomer), 35.4 (t, *J*=19.5 Hz, C-2 of *E*-isomer), 129.0 (2C, t, *J*=24.4 Hz, C-2′, 6′), 129.1 (2C, t, *J*=23.9 Hz, C-3′, 5′),

135.0 (s, C-1′ of *E*-isomer), 135.2 (s, C-1′ of *Z*-isomer), 150.5 (t, *J*=26.8 Hz, C-1 of *E*-isomer), 150.7 (t, *J*=26.8 Hz, C-1 of *Z*-isomer). The deuterium ratio of [1,2,2′,3′,4′,5′,6′-$^2H_7$]-**7** was not estimated. The ratio (3:1) of *Z*- to *E*-isomers was determined on the basis of the ^1H NMR spectrum of **7**, prepared from 1-nitrostyrene[30] according to the method mentioned above. ^1H NMR (500 MHz, CD_3OD) of **7**; δ_H: 3.54 (0.25H, d, *J*=12.0 Hz H-2 of *E*-isomer), 3.75 (0.75H, d, *J*=10.0 Hz, H-2 of *Z*-isomer), 6.89 (0.75H, t, *J*=10.0 Hz, H-1 of *Z*-isomer), 7.54 (0.25H, t, *J*=12.0 Hz, H-1 of *E*-isomer), 7.20–7.36 (5H, H-2′-6′).

3.2. Plant materials

Rosa damascena Mill. and *Rosa* 'Hoh-Jun' plants were grown on the University Farm, Faculty of Agriculture, Shizuoka University, Japan under natural conditions in 5-l pots, watered daily and fed weekly with a nutrient solution. Each plant bore 30–50 (*R. damascena*) and 1–4 (*R.* 'Hoh-Jun') flowers at various stages. *R.* 'Hoh-Jun' is a cultivar that produces flowers throughout the year, whereas *R. damascena* produces flowers from the end of April until mid-May in Shizuoka, Japan. Plants were acclimatized for 7 days to a constant temperature of 22±2 °C and a 12 h/12 h light/dark (LD) cycle in a controlled environment growth room with a natural light source (approximately 300 μmol m^{-2} s^{-1}). In subsequent experiments, plants were acclimatized to 22±2 °C, 75±5% humidity and 12 h photoperiods (300 μmol m^{-2} s^{-1}, provided by fluorescent lamps) in a controlled environment growth room.

The stages of floral growth are defined as follows: stage 1, immature flowers, sepals tightly closed; stage 2, sepals retracting, petal whorl tightly closed, petals beginning to lighten in color; stage 3, commencement of petal unfurling (4 days after stage 1), sepals fully retracted, outer petal whorl beginning to loosen; stage 4, outer petal whorl opened, inner petal whorl beginning to loosen; stage 5, inner petal whorl partly opened but reproductive organs not yet visible; stage 6, inner and outer whorls open, reproductive organs not visible; stage 7, full bloom flower, inner and outer whorls open, reproductive organs visible; stage 8, inner and outer whorls completely open, 30 h (*R. damascena*) or 5 days (*R.* 'Hoh-Jun') after stage 3; stage 9, senescence stage, anthers dried and darkened, petals pale.

3.3. Phenyl-*N*-glucosyl-acetamidiumbromide (8) feeding in *R. damascena* Mill. flowers

Feeding studies done in April to May of 2002 and 2003 were conducted on opening flowers at stage 3 in intact *R.* 'Hoh-Jun' plants as described above. A thin needle with a cotton thread (50 mm in length) was inserted at 2 p.m. on day 0 through the top of the ovary, just above the ovules. This thread acted as a wick enabling the absorption of an aqueous solution of **8** (10 mM, 300 μl) from an Eppendorf tube attached to the stem. A control group was evaluated in parallel in which water was fed instead of **8**. The total amount of **8** absorbed by the plant was not evaluated. Three treated flower heads at 6, 12, 16, 18, 20, 24, 30, 36, and 42 h after feeding startted were detached just below the ovary of the flower. The calyx was removed and the petal parts of each flower head were crushed in liquid nitrogen and stored at −80 °C until use.

3.4. [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-2-Phenylethyl β-D-glucopyranoside (3) feeding in *R.* 'Hoh-Jun' flowers

A solution of [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**3** (3.4 mM, 1500 μl) was fed to the flowers at stage 3 in intact *R.* 'Hoh-Jun' plants as described above. The floral scent compounds emitted from the flowers were trapped by the dynamic head space method using automatic sampling system equipped with a time controller and six air-valves developed by the authors. A Tenax-TA column (150 mg, 3 mm i.d. ×100 mm) was used as an absorbent with an air at flow rate of 500 ml/min. Scent compounds were eluted from the column with CH_2Cl_2–diethyl ether (1:1, v/v) and concentrated at 40 °C. The concentrated materials were directly analyzed by GC-SIM.

3.5. [1,2,2,2′,3′,4′,5′,6′-$^2H_8$] L-Phenylalanine (1), [$^2H_8$]-1 feeding in *R.* 'Hoh-Jun' flowers

Feeding studies done in October and November 2002 were conducted on flowers at stage 3 in intact *R.* 'Hoh-Jun' plants as described above. An aqueous solution of [1,2,2,2′,3′,4′,5′,6′-$^2H_8$]-**1** at each concentration, 1.1, 2.1, 4.3, 8.7 and 17.3 mM, 1.5 ml was fed as described above. The total amount of [$^2H_8$]-**1** absorbed by the plant was not evaluated. Three of the treated flower heads were detached just below the ovary of the flower at 48 h after feeding started. The petal parts were crushed in liquid nitrogen and stored −80 °C. [^2H_n]-**2/2** and [^2H_n]-**3/3** were extracted by pentane and ethyl acetate, respectively. The ratio of [^2H_n]-**2/2** and [^2H_n]-**3/3** in the tissues were estimated from the GC-SIM data.

In May, 2003, [$^2H_8$]-**1** (17.3 mM, 1.5 ml) was fed to five flowers at stage 3 in intact *R.* 'Hoh-Jun' plants as described above. The flowers were detached at 6, 12, 24, 48, and 72 h after feeding started and were treated as described above. Changes in the ratio of [$^2H_8$]-**2**/[$^2H_7$]-**2** and [$^2H_8$]-**3**/[$^2H_7$]-**3** during the unfurling process were estimated from the GC-SIM data.

3.6. [1,1,2,2,2′,3′,4′,5′,6′-$^2H_9$]-2-Phenylethylamine (6) feeding in *R.* 'Hoh-Jun' flowers

In May, 2003, [1,1,2,2,2′,3′,4′,5′,6′-$^2H_9$]-**6** (23 mM solution, pH 6.8 as HCl salt, 1500 μl) was fed to five immature flowers at stage 3 for 96 h until the full bloom stage (stage 7) of *R.* 'Hoh-Jun' by the method just described above. The flowers were detached at 96 h after feeding started and were treated as described in Section 3.3.

3.7. [1,2,2′,3′,4′,5′,6′-$^2H_7$]-2-Phenylacetaldoxime (7) feeding in *R.* 'Hoh-Jun' flowers

In June, 2003, 1 μl of [1,2,2′,3′,4′,5′,6′-$^2H_7$]-**7** (14 mM of DMSO solution) was injected to the top of the ovary, just above the ovules of three immature flowers at stage 3. The flowers were detached at 24 h after injection and treated as described in Section 3.3.

3.8. Extraction of the volatile compounds and glycoconjugates

A portion (equivalent to one flower head) of each type of frozen flower petal was extracted twice with pentane (20 ml each) and then twice with EtOAc (20 ml each) in a microwave oven according to the method of Oka et al.[11] Ethyl octanoate (20 μg) and phenyl β-D-glucopyranoside (20 μg) were used as internal standards in the GC, GC-MS and GC-SIM analyses. The pentane extract was concentrated and directly analyzed by GC-MS and GC-SIM revealing the presence of **2** and [^2H_n]-**2**. The EtOAc extract was evaporated in vacuo and the resulting residue was re-dissolved in a citrate buffer (10 mM, pH 6.0) and hydrolyzed by a mixture of β-glucosidase (2500 units) and naringinase (3000 units) yielding the volatile compounds. After extraction with an azeotropic mixture (bp 38 °C) of pentane–CH_2Cl_2 (2:1),[12] the resulting extract was analyzed by GC-MS and GC-SIM to evaluate the amount of **2** and [^2H_n]-**2** as an aglycone part of **3** and [^2H_n]-**3**, respectively. To quantify **3** in the flower tissues, the ethyl acetate extract was directly analyzed for pertrifluoroacetates (TFA) derivatives by GC.

3.9. GC analyses

Ethyl acetate extract (from 100 to 500 mg of fresh flower petals) was concentrated and analyzed by GC after conversion into TFA-derivatives by treatment with *N*-methyl-bis(trifluoroacetamide-pyridine. A HITACHI G-3000 gas chromatograph was used for the analyses. A TC-1 column (df=0.25 mm), dimensions 0.25 mm i.d.×30 m, was used; the column temperature was raised from 150 to 280 °C (increment 2 °C/min); the injection temperature was 230 °C. For identification and quantitative analysis, **3** was analyzed as its TFA-derivative, and 0.1 μl of the reaction mixture was injected onto the GC column. Identification and quantification were carried out based on the peak area and the relative retention time (t'_R=2.957 for tetra *O*-TFA-**3**) relative to phenyl β-D-glucopyranoside-tetra-*O*-TFA, which was used as an internal standard (t_R=11.32 min).

3.10. GC-MS and GC-SIM analyses

The GC-MS analysis was conducted with a SHIMADZU QP505A gas chromatograph-mass spectrometer equipped with a SHIMADZU GC-17A. The column was a 30 m TC-WAX type with 0.25 mm i.d.×30 m; the column temperature was elevated from 100 to 170 °C (2 °C/min); the injector temperature was 250 °C; the ionizing voltage was 70 eV; the scanning speed was 0.5 scan/s with the range of *m/z* 40–250. 1 μl of each concentrate of solvent extract from petal tissues and of eluate from a TENAX columns was injected to the GC-MS. The identification of **2** and [^2H_n]-**2** was established by comparing their MS spectra with authentic samples as previously described by Watanabe et al.[18] Quantitative analyses of **2** and [^2H_n]-**2** were carried out from the SIM traces at *m/z* 130 [M^+] of [$^2H_8$]-**2**, *m/z* 129 [M^+] of [$^2H_7$]-**2**, *m/z* 128 [M^+] of [$^2H_6$]-**2**, *m/z* 98 [$C_7D_7^+$], *m/z* 97 [$C_7D_6H^+$], *m/z* 96 [$C_7D_5H_2^+$] and at *m/z* 122 [M^+] and *m/z* 91 [$C_7H_7^+$] for **2**. GC-SIM conditions were the same as mentioned above. Authentic [1,1,2,2′,3′,4′,5′,6′-$^2H_8$]-**2** (MW 130) and **2** were detected at t_R=18.23 and 18.48 min, respectively.

3.11. Estimation of β-glucosidase activity in the petal tissues during unfurling process of *R. damascena* Mill

2 ml of citrate buffer (50 mM, pH 6.0, containing 5 mM EDTA 2 Na, 2 mM dithiothreitol, 10 mM ascorbic acid, and 1% Triton X-100) was added to 0.1 g of liquid nitrogen powder (prepared from flower petals at the onset of unfurling) and stirred for 30 min at 4 °C, and was then centrifuged at 2000*g* for 10 min at 4 °C. The supernatant was passed through a membrane filter (0.45 μm) to give a crude enzyme solution. The β-glucosidase activity of the crude enzyme solution was evaluated as follows: (1) 50 mM of **3**, 100 μl in citrate buffer, plus crude enzyme, 200 μl; (2) citrate buffer, 100 μl, plus crude enzyme, 200 μl; (3) 50 mM of **3**, 100 μl in the citrate buffer plus citrate buffer, 200 μl. The three mixtures were incubated for 360 min at 37 °C, and the **2** released from **3** was extracted 5 times with pentane–CH_2Cl_2 (2:1) in the presence of ethyl decanoate (10 μg). The combined extract was concentrated and analyzed by GC-MS. The conditions were the same as described in Section 3.10. The activity was expressed as the amount of **2** (μmol) released from **3** in the initial 60 min of the incubation period. The inhibitory effect of **8** (6.0×10^{-9}, 1.0×10^{-8}, 6.0×10^{-8}, 1.0×10^{-7}, and 6.0×10^{-7} mol) toward the crude enzymes prepared from flower petals (0.1, 0.2, and 0.4 g fresh weight of the petals) at stage 6 was examined as described above. The hydrolysis of **3** was inhibited by 95% at a dose of 6.0×10^{-7} mol of **8**.

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References and notes

1. Knudsen, J. T.; Tollsten, L.; Bergström, L. G. *Phytochemistry* **1993**, *33*, 253–280.
2. Schiestl, F. P.; Ayasse, M.; Paulus, H. F.; Erdmann, D.; Francke, W. *J. Chem. Ecol.* **1997**, *23*, 2881–2895.
3. Borg-Karlson, A. K.; Valterova, I.; Nilsson, L. A. *Phytochemistry* **1994**, *46*, 1169–1172.
4. Ervik, F.; Tollsten, L.; Knudsen, J. T. *Plant Syst. Evol.* **1999**, *217*, 279–297.
5. Odell, E.; Raguso, R. A.; Jones, K. N. *Am. Midl. Nat.* **1999**, *142*, 257–265.
6. Jurgens, A.; Webber, A. C.; Gottsberger, G. *Phytochemistry* **2000**, *55*, 551–558.
7. Miyake, T.; Yamaoka, R.; Yahara, T. *J Plant Res.* **1998**, *111*, 199–205.
8. Omura, H.; Honda, K.; Hayashi, N. *J. Chem. Ecol.* **2000**, *26*, 655–666.

9. Francis, M. O.; Banthorpe, D. V.; Le Patourel, G. N. J. *Nature* **1970**, *228*, 1005–1006.
10. Francis, M. O. J.; O'Connell, M. *Phytochemistry* **1969**, *8*, 1705–1708.
11. Banthorpe, D. V.; Le Patourel, G. N. J.; Francis, M. J. O. *Biochem. J.* **1972**, *130*, 1045–1054.
12. Akermann, I. E.; Banthorpe, D. V.; Fordham, W. D.; Kinder, J. P.; Poots, I. *J. Plant Physiol.* **1989**, *134*, 567–572.
13. Loughrin, J. H.; Hamilton-Kemp, T. R.; Andersen, R. A.; Hildebrand, D. F. *Physiol. Plant* **1991**, *83*, 492–496.
14. Oka, N.; Ohishi, H.; Hatano, T.; Hornberger, M.; Sakata, K.; Watanabe, N. *Z. Naturforsch.* **1999**, *54c*, 889–895.
15. Reuveni, M.; Sagi, Z.; Evnor, D.; Hetzroni, A. *Plant Sci.* **1999**, *147*, 19–24.
16. Loughrin, J. H.; Hamilton-Kemp, T. R.; Burton, H. R.; Andersen, R. A.; Hildebrand, D. F. *Phytochemistry* **1992**, *31*, 1537–1540.
17. Jakobsen, H. B.; Christensen, L. P. *Plant Cell Env.* **2002**, *25*, 773–781.
18. Watanabe, S.; Hayashi, K.; Yagi, K.; Asai, T.; MacTavish, H.; Picone, J.; Turnbull, C.; Watanabe, N. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 943–947.
19. Guo, W.; Hiratake, J.; Ogawa, K.; Yamamoto, M.; Ma, S. J.; Sakata, K. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 467–470.
20. Inoue, K.; Hiratake, J.; Mizutani, M.; Takada, M.; Yamamoto, M.; Sakata, K. *Carbohydr. Res.* **2003**, *338*, 1477–1490.
21. Picone, M. J.; Clery, R. A.; Watanabe, N.; MacTavish, H. S.; Turnball, C. G. N. *Planta*, in press.
22. Albertazzi, E.; Cardillo, R.; Servi, S.; Zucchi, G. *Biotech. Lett.* **1994**, *16*, 491–496.
23. Robins, R. J.; Woolley, J. G.; Ansarin, M.; Eagles, J.; Goodfellow, B. J. *Planta* **1994**, *194*, 86–94.
24. Dickinson, J.; Salgado, L. E.; Hewlins, J. E. *J. Biol. Chem.* **2003**, *278*, 8028–8034.
25. Walsch, C. *Enzymatic Reaction Mechanisms*; W.H. Freeman and Co: New York, 1978; Chapter 14, pp 451–454.
26. Hazen, S.; d'Avignon, A.; Anderson, M. M.; Hsu, F. F.; Heinecke, J. W. *J. Biol. Chem.* **1998**, *273*, 4997–5005.
27. Saijo, R. *Jpn. Agric. Res. Q.* **1973**, *7*, 202–207.
28. Wittstock, U.; Halkier, B. A. *J. Biol. Chem.* **2000**, *275*, 14659–14666.
29. Kaiser, R. *J. Ess. Oil Res.* **1991**, *3*, 129–146.
30. Campos, P. J.; Garcia, B.; Rodriguez, M. A. *Tetrahedron Lett.* **2000**, *41*, 979–982.
31. Sera, A.; Yamauchi, H.; Yamada, H.; Itoh, K. *Synlett* **1990**, 477–478.