Conversion of Citronellyl Diphosphate and Citronellyl β-D-Glucoside into Rose Oxide by *Pelargonium graveolens*

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Deuterium-labeled citronellyl diphosphate and citronellyl β-D-glucoside were fed to *Pelargonium graveolens*. Both precursors were converted into *cis-/trans*-rose oxide. Citronellyl diphosphate is more effectively converted into rose oxide. The phloem exudate of *P. graveolens* was analyzed after enzymatic cleavage of the glucosidated and phosphorylated volatiles. It could be shown that glucosidically bound citronellol is translocated in the plant. Phosphorylated citronellol could not be detected.

Keywords: *Pelargonium graveolens; Geraniaceae; stable isotope labeling; monoterpenes; rose oxide; citronellyl diphosphate; citronellyl β-D-glucoside*

INTRODUCTION

Glycosidically bound volatiles have attracted much attention since the first detection of monoterpenol glucosides in rose petals in 1969 (Francis and Allock, 1969). Already in 1991 ~200 aglycon structures have been found in ~150 plant species belonging to 35 different families (Stahl-Biskup et al., 1993). Up to now glycosidically bound volatiles have been detected in almost 50 plant families (Winterhalter and Skouroumounis, 1997). The role of glycosidically bound volatiles in plants is still discussed. They are considered as accumulation and storage forms as well as transport forms for hydrophobic substances (Stahl-Biskup, 1987; Stahl-Biskup et al., 1993). Croteau and co-workers could show that in sage (*Salvia officinalis*) and peppermint (*Mentha piperita*) radiolabeled monoterpene substrates were converted to water soluble glycosidic transport derivatives, which were catabolized and reutilized in the biosynthesis of phytosterols and acyl lipids (Croteau, 1988). Glycosidation is also considered as a protective mechanism to prevent the volatile alcohols from destroying membranes (Stahl-Biskup, 1987). In summary, it can be stated that glycosidically bound volatiles are involved in different processes and cannot be ascribed to an exclusive role. Further bound volatiles are the allylic terpene diphosphates, which play the central role in isoprenoid metabolism (Croteau, 1987). Recently, phosphorylated monoterpenes were identified in green tea leaves by the combination of LC-MS/MS and HRGC-MS (Ney et al., 1996). Because the free terpene alcohols are toxic to the plant, it is reasonable to apply glucosidated or phosphorylated terpene alcohols in feeding experiments with labeled precursors. It was recently shown that deuterium-labeled citronellyl glucosides, if administered to *Pelargonium graveolens* or *Pelargonium radens* by the cut-stem method, are translocated and converted into *cis-/trans*-rose oxide (**1**−**4**, see Figure 1) (Wüst et al., 1996). The mechanism of this reaction has

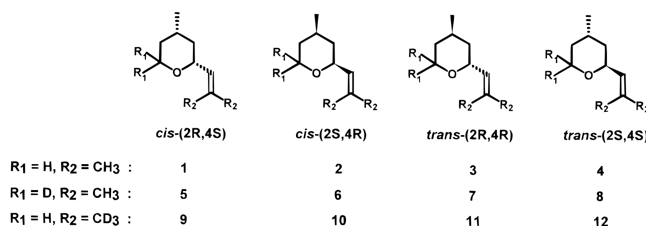

Figure 1. Stereoisomers of rose oxide and its isotopomers.

been investigated very recently using mixed labeled citronellol isotopomers and labeled menthocitronellol (Wüst et al., 1998a,b). With regard to the biogenesis of rose oxide in *Pelargonium* species, it is of interest to compare citronellyl glucoside and citronellyl diphosphate as precursors of rose oxide. Therefore, this paper deals with the synthesis of different specifically deuterium-labeled citronellyl glucosides and citronellyl diphosphates and the occurrence of these bound forms of citronellol in the plant and compares citronellyl glucoside and citronellyl diphosphate in relation to this conversion into rose oxide.

EXPERIMENTAL PROCEDURES

Plant Material. Young plants of *P. graveolens* were kindly provided by Gartenbau Stegmeier, Essingen, Germany.

NMR. The spectra were recorded on a Bruker AMX 500: ^{1}H, 500 MHz, $CDCl_3$/TMS or D_2O/DSS; ^{13}C, 125 MHz, $CDCl_3$/TMS; ^{31}P, 202.46 MHz, D_2O, 80% aqueous H_3PO_4 as external standard.

Synthesis of 3,7-Dimethyl-[1,1-$^{2}H_2$]-6-octen-1-yl *p*-Toluenesulfonate (13). 3,7-Dimethyl-[1,1-$^{2}H_2$]-6-octen-1-ol was synthesized as previously described (Wüst et al., 1998a). The tosylate was prepared according to the method of Davisson et al. (1986); 4.07 mmol (644 mg) of the labeled alcohol gave 2.89 mmol (908 mg) of **13**: ^{13}C NMR ($CDCl_3$/TMS, ^{1}H decoupling) δ 17.6, 19.0, 21.6, 25.2, 28.8, 35.4, 36.7, 68.4 (quintet, J = 23 Hz, C-1), 124.3, 127.8, 129.8, 131.4, 133.2, 144.6.

Synthesis of 3,7-Dimethyl-[1,1-$^{2}H_2$]-6-octen-1-yl Diphosphate (14). 14 was prepared according to the method of Davisson et al. (1986) and purified by crystallization as Li salt according to the method of Bunton et al. (1972); 1.84 mmol (580 mg) of **13** gave 0.35 mmol (117 mg) of **14**: ^{1}H NMR (D_2O/

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DSS) δ 0.88 (3H, d, *J* = 6.6 Hz, H-9), 1.1−1.38 (2H, m, H-4), 1.42 (1H, dd, *J* = 13.2, 8.1 Hz, H-2a), 1.61 (3H, s, H-8), 1.67 (3H, s, H-10), 1.5−1.7 (2H, m, H-3, H-2b), 1.9−2.1 (2H, m, H-5), 5.22 (1H, m, H-6); ^{31}P NMR (D_2O, pH 8) δ −2.38 (1P, d, *J* = 20.4 Hz), −6.17 (1P, d, *J* = 20.4 Hz).

Synthesis of 3,7-Dimethyl-[8,8,8,10,10,10-$^2H_6$]-6-octen-1-yl β-D-Glucopyranoside (15). 3,7-Dimethyl-[8,8,8,10,10,-10-$^2H_6$]-6-octen-1-ol was synthesized as previously described (Wüst et al., 1998c). For glucosidation the Koenigs−Knorr procedure was used (Paulsen et al., 1985); 1.56 mmol (253 mg) of the labeled alcohol gave 0.31 mmol (100 mg) of **15**. Diastereomeric ratio of *R*-**15** and *S*-**15** was 57/43 (determined by enzymatic cleavage and enantioselective GC of liberated citronellol): ^{1}H NMR ($CDCl_3$/TMS) δ 0.89 (3H, d, *J* = 6.3 Hz, H-9), 1.1−1.4 (2H, m, H-4), 1.4−1.8 (3H, m, H-2, H-3), 1.85−2.1 (2H, m, H-5), 3.25−4.1 (12H, m), 4.31 (1H, d, *J* = 7.7 Hz, H-1′), 5.09 (1H, t, *J* = 7.1 Hz, H-6).

Reference Compounds. *cis*-(2*R*,4*S*)/*trans*-(2*S*,4*S*)-Rose oxides (**1**, **4**) (enantioselective GC: ee = 65%, *cis/trans* = 75:25) were obtained from Dragoco, Holzminden, Germany.

(±)-*cis*-/*trans*-2-(2-Methyl-1-propenyl)-4-methyl[6,6-$^2H_2$]tetrahydropyran (**5**−**8**) and (±)-*cis*-/*trans*-2-(2-[2,2,2-$^2H_3$]methyl-1-[3,3,3-$^2H_3$]propenyl)-4-methyltetrahydropyran (**9**−**12**) were prepared as previously described (Wüst et al., 1998c).

Enzymatic Cleavages. *Enzymatic Cleavage of **15** with β-D-Glucosidase.* β-D-Glucosidase (0.7 mg; from sweet almonds, EC 3.2.1.21, 30 units/mg of solid, Sigma) and 20 μg of **15** were mixed with 1 mL of a citrate buffer (pH 5.5) in a 2 mL vial and covered with a layer of 200 μL of pentane. The vial was sealed and incubated for 12 h at 37 °C. The enantiomeric ratio of the liberated citronellol in the pentane layer was determined by enantio-GC.

*Enzymatic Cleavage of **14** and **15** with β-D-Glucosidase and Phosphatase.* β-D-Glucosidase (0.5 mg; see above) was mixed with 1 mL of a citrate buffer (pH 5.5). Five hundred microliters of this solution was mixed with 10 μg of **14** and 10 μg of **15** in a 2 mL vial and covered with a layer of 400 μL of pentane. The vial was sealed and incubated for 24 h at room temperature. The other 500 μL of the glucosidase solution were mixed with 10 μg of **14**, 10 μg of **15**, and 10 μL of phosphatase (from sweet potato, EC 3.1.3.2., 0.675 unit/μL, Sigma) in a 2 mL vial and covered with a layer of 400 μL of pentane. The vial was sealed and incubated for 24 h at room temperature. The ratio of the liberated aglycons in the pentane layer was determined by GC.

*Enzymatic Cleavage of **14** and **15** with Phosphatase.* Ten microliters of phosphatase (see above) was dissolved in 500 μL of citrate buffer containing 0.2 M EDTA and 0.2 M gluconic acid δ-lactone. The solution was mixed with 10 μg of **14** and 10 μg of **15** in a 2 mL vial and covered with a layer of 400 μL of pentane. The vial was sealed and incubated for 24 h at room temperature. The ratio of the liberated aglycons in the pentane layer was determined by GC.

Enzymatic Cleavage of Phloem Exudates with β-D-Glucosidase. Two hundred microliters of the phloem exudates obtained by using the EDTA method (see below) was incubated with 0.5 mg of glucosidase (see above) for 24 h at room temperature in a sealed 2 mL vial. A blank experiment was carried out without the enzyme. After the incubation, the headspace of the vial was analyzed using SPME and MDGC-MS (see below).

Enzymatic Cleavage of Phloem Exudates with Phosphatase. Two hundred microliters of the phloem exudates obtained by using the EDTA method (see below) containing 0.2 M EDTA and 0.2M gluconic acid δ-lactone was incubated with 30 μL of phosphatase (see above) for 24 h at room temperature in a sealed 2 mL vial. A blank experiment was carried out without the enzyme. After the incubation, the headspace of the vial was analyzed using SPME and MDGC-MS (see below).

GC. The GC analyses of the enzymatic cleavage experiments were performed on a Carlo Erba Strumentazione HRGC 5160 Mega Series. An FS capillary column (30 m × 0.25 mm) was used: film thickness, 0.38 μm PS-268; carrier gas, hydrogen, 95 kPa; split, 40 mL/min; injector, 240 °C; detector, FID 240 °C; temperature program, 60 °C isothermal for 5 min, raised at 2.5 °C/min to 200 °C; injection volume, 1 μL.

Enantio-GC. The enantio-GC analysis of the enzymatic cleavage experiments was performed on a Hewlett-Packard 5890 Series II. A Duranglas capillary column (26 m × 0.23 mm) was used: film thickness, 0.1 μm of 50% heptakis(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin in OV-1701-vi; carrier gas, hydrogen, 105 kPa; split, 17 mL/min; injector temperature, 220 °C; detector, FID 240 °C; temperature program, 40 °C isothermal for 5 min, raised at 1.0 °C/min to 200 °C; injection volume, 1 μL.

MDGC-MS. The multidimensional gas chromatography−mass spectrometry analysis (MDGC-MS) of the solid-phase microextraction (SPME) headspace extracts of the phloem exudates was performed with a Siemens Sichromat 2-8, equipped with independent column oven programs and a live-T-switching device. The main column was coupled to the transfer line of a Finnigan-MAT ITD 800, using an open split interface. Precolumn conditions were as follows: Duranglass capillary (30 m × 0.25 mm), coated with a 0.38 μm film of PS-268; carrier gas, hydrogen, 120 kPa; split, 25 mL/min; injector temperature, 220 °C; detector, FID, 280 °C; oven temperature, 50 °C (5 min isothermal), raised at 3 °C/min to 270 °C. Main column conditions were as follows: FS capillary column (30 m × 0.32 mm), coated with a 0.5 μm film of 30% heptakis-(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin in SE-52; carrier gas, hydrogen, 68 kPa; oven temperature, 50 °C (30 min isothermal), raised at 2 °C/min to 200 °C; transfer line, 250 °C; open split interface, 250 °C; helium sweeping flow, 1 mL/min; ion trap manifold, 230 °C; EI, 70 eV. The reference compounds and SPME headspace extracts were analyzed in full scan mode (40−250 amu).

Enantio-MDGC-MS. The enantio-multidimensional GC-MS analysis (enantio-MDGC-MS) of the solid-phase microextraction (SPME) headspace extracts, synthetic products, and reference compounds was performed with a Siemens Sichromat 2, equipped with independent column oven programs and a live-T-switching device. The main column was coupled to the transfer line of a Finnigan-MAT ITD 800 (see above). Precolumn conditions were as follows: Duranglass capillary (28 m × 0.23 mm), coated with a 0.23 μm film of PS-268; carrier gas, hydrogen, 125 kPa; split, 25 mL/min; injector temperature, 220 °C; detector, FID, 250 °C; oven temperature, 110 °C (30 min isothermal), raised at 2 °C/min to 250 °C. Main column conditions were as follows: Duranglass capillary (30 m × 0.23 mm), coated with a 0.23 μm film of 50% heptakis(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin in PS-268 (polydimethylsilaxane); carrier gas, hydrogen, 100 kPa; oven temperature, 60 °C (25 min isothermal), raised at 2 °C/min to 180 °C. The reference compounds and synthetic products were analyzed in full scan mode (40−250 amu). The genuine and deuterium-labeled rose oxides **1**−**12** were detected in SIM mode (selected ion monitoring): *m*/*z* 139−142. Live-T cuttings *cis*-/-*trans*-rose oxides (**1**−**12**): 10.00−12.00 min. Order of elution on main column: *cis*-rose oxide, **9**, **5**, **1** (2*R*,4*S*); **10**, **6**, **2** (2*S*,4*R*); *trans*-rose oxide **11**, **7**, **3** (2*R*,4*R*); **12**, **8**, **4** (2*S*,4*S*).

Administration of the Labeled Precursors. A solution of **14** and **15** with a concentration of 0.5 mg/mL (1.5 mM) of each precursor was prepared by dissolving the same amounts of **14**, **15**, and Tween 20 in water; 200 μL of this solution and two pieces of the leaf blade of *P. graveolens* (weight = 100−200 mg) were incubated at room temperature for 24 h with exclusion of light in a sealed 2 mL vial. After incubation, the essential oil evaporating from the glandular trichomes was analyzed by headspace SPME (see below).

Collection of Phloem Exudates. For collecting phloem exudates the EDTA method was applied (King and Zeevaart, 1974). A cut stem of *P. graveolens* with several leaves (weight ∼20 g) was recut under water and immersed in a solution of 20 mM EDTA in phosphate buffer (pH 7). The cut stem was kept in the dark for 2 h at room temperature. The cut stem was rinsed with water and placed in fresh water (10 mL) for 24 h in the dark at room temperature. The phloem exudates of four cut stems were collected by using this method, and the water was removed under reduced pressure. The residue was extracted with 20 mL of pentane and dissolved in 2 mL of citrate buffer (pH 5.5). After enzymatic cleavage (see above),

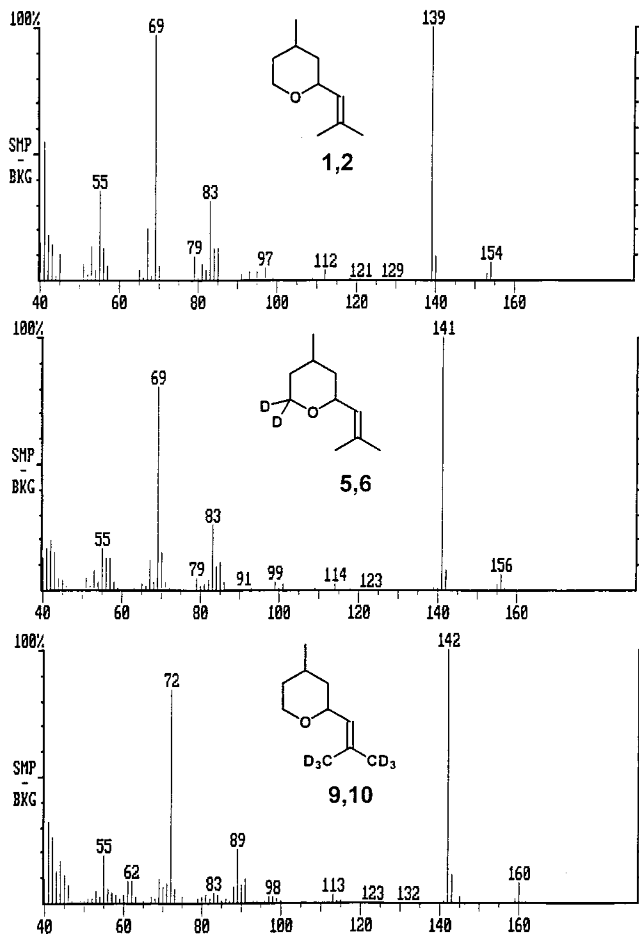

Figure 2. Mass spectra of unlabeled and labeled rose oxide.

the phloem exudates were analyzed using SPME (see below) and MDGC-MS (see above).

SPME. An SPME fiber holder for manual use equipped with a fused silica fiber coated with poly(dimethylsiloxane) (film thickness = 100 μm) was used (both Supelco, Munich, Germany). For headspace sampling the septum of the vial containing the pieces of leaf blade or the phloem exudate was pierced and the fiber was exposed for 3 min to the headspace. For thermal desorption, the SPME fiber remained in the injector for 3 min. Splitless injection mode was used, the split valve being opened after 2 min.

RESULTS AND DISCUSSION

To compare the activity of citronellyl glucoside and citronellyl diphosphate as precursors of *cis-/trans*-rose oxide, compounds **14** and **15** (see Figure 4) were prepared by phosphorylation and glucosidation of two different specifically labeled citronellol isotopomers. The starting material used for their synthesis was commercially available citronellic acid, which was not racemic (*R*/*S* = 57:43). To verify whether an enantiodiscrimination takes place during the glucosidation reaction, the resulting mixture of the diastereomeric citronellyl glucosides was quantitatively cleaved by emulsin (Günata et al., 1990). The enantiomeric ratio of the liberated citronellol was determined by enantio-GC and was found to be identical with the enantiomeric ratio of the starting material. Hence, no enantiodiscrimination takes place during the glucosidation. The mass spectra of the corresponding labeled *cis*-rose oxides are shown in Figure 2. The genuine rose oxides **1**−**4** can be detected on mass lane *m*/*z* 139. The dideuterated rose

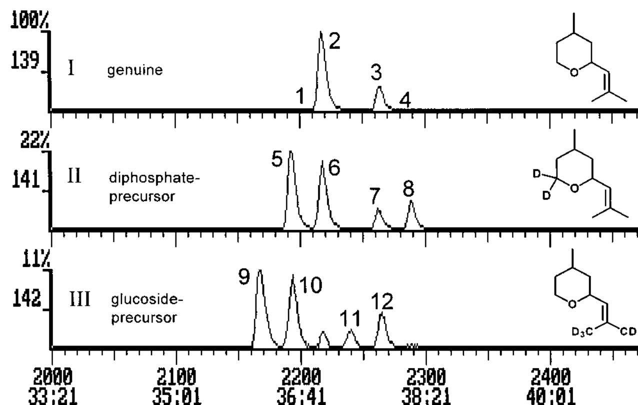

Figure 3. Main column chromatogram of a headspace extract of *P. graveolens* fed with a 1:1 mixture of **14** and **15** detected in SIM mode (*m*/*z* 139−142): mass lane I, *m*/*z* 139; mass lane II, *m*/*z* 141; mass lane III, *m*/*z* 142.

oxides **5**−**8** resulting from the diphosphate precursor **14** can be detected on mass lane *m*/*z* 141 without any superimposition from the genuine one. The hexadeuterated rose oxides **9**−**12** resulting from the glucosidic precursor **15** can be detected on mass lane *m*/*z* 142 without any superimposition from the genuine one. Furthermore, the hexadeuterated rose oxides show considerably shorter retention times than the genuine ones (see Figure 3, mass lanes *m*/*z* 139 and 142). This inverse isotope effect is proportional to the number of deuterium atoms in the molecule (Matucha et al., 1991). Due to this shift of the rentention times, the isotopomers **5** and **10** as well as **7** and **12** are coeluting (see Figure 3, mass lanes *m*/*z* 141 and 142). Hence, if a mixture of **14** and **15** is fed to the plant, the peak areas of the hexadeuterated rose oxides **10** and **12** have to be corrected because the mass spectra of the dideuterated rose oxides **5**−**8** show a small peak at *m*/*z* 142 with relative intensities of 8.3 (*cis*-**5**) and 8.9 (*trans*-**7**), respectively (Wüst et al., 1998a).

A solution containing **14** and **15** equal in concentration was fed to the plant by incubating leaf disks with the feeding solution in septum-sealed 2 mL vials for 24 h in the dark. After the incubation time, the headspace of the vials was analyzed using SPME and enantio-MDGC-MS. Figure 3 shows the main column chromatogram detected in SIM mode (*m*/*z* 139−142). Mass lane *m*/*z* 139 shows the genuine *cis*- and *trans*-rose oxides of high enantiomeric purity (see Table 1). The diastereomeric ratio of the 4*R* (**2**, **3**) and 4*S* (**1**, **4**) configured rose oxides is approximately 3:1 (see Table 1). Mass lane 141 shows the rose oxides resulting from the diphosphate precursor **14**. The enantiomeric ratios of *cis*- and *trans*-rose oxide are almost identical with the enantiomeric ratio of the fed precursor **14**. This shows that the cyclization of the precursor to rose oxide takes place with retention of the absolute configuration of the primary stereocenter of the citronellyl moiety. The diastereomeric ratios of the rose oxides are approximately 3:1 and correspond with the genuine ones. Similar results are obtained from the hexadeuterated rose oxides resulting from the glucoside precursor **15** and are in correspondence with earlier results when only the glucoside precursor was fed to the plant (Wüst et al., 1996).

The ratio of the generated dideuterated and hexadeuterated rose oxides is approximately 2:1 (1.9 ± 0.37), which shows that the phosphorylated citronellyl precur-

Table 1. Enantiomeric and Diastereomeric Ratios of Rose Oxide in Percent of *P. graveolens* Fed with a 1:1 Mixture of 14 and 15

	enantiomeric ratio of rose oxides (%)		diastereomeric ratio of rose oxides (%)	
m/z	*cis*-(2*R*,4*S*)/*cis*-(2*S*,4*R*)	*trans*-(2*R*,4*R*)/*trans*-(2*S*,4*S*)	*cis*-(2*S*,4*R*)/*trans*-(2*R*,4*R*)	*cis*-(2*R*,4*S*)/*trans*-(2*S*,4*S*)
139 (genuine)	1:99 (0.2)[a]	98:2 (0.4)	76:24 (0.3)	70:30 (7.7)
141 (labeled)	57:43 (1.3)	42:58 (2.1)	77:23 (1.4)	76:24 (0.9)
142 (labeled)	60:40 (0.6)	36:64 (2.2)	78:22 (0.9)	75:25 (1.4)

[a] Standard deviations are given in parentheses; $n = 5$.

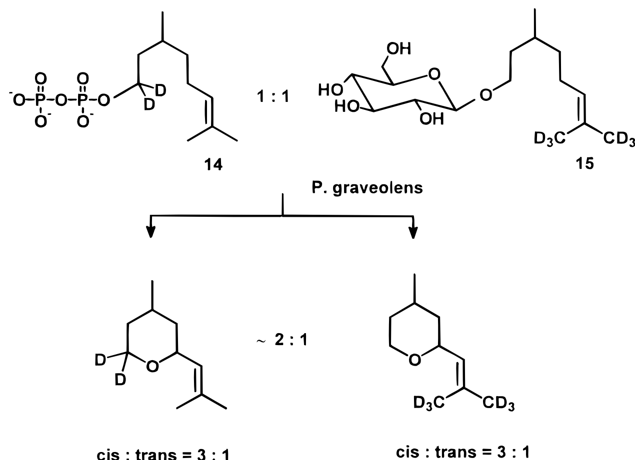

Figure 4. Conversion of **14** and **15** into rose oxide by *P. graveolens*.

sor **14** is more effectively converted into rose oxide than the glucosidated one (see Figure 4).

This fact may be due to the better solubility of citronellyl diphosphate in water and, therefore, it is perhaps more easily translocated in the plant. Another possibility is the faster hydrolysis of the diphosphate. In this context we have analyzed the phloem exudate of the plant, which was obtained by using the EDTA method (King and Zeevaart, 1974). The phloem exudate was incubated with β-glucosidase from sweet almonds and phosphatase from sweet potato, respectively. The liberated compounds were identified by SPME and MDGC-MS. The enzymes were tested before by using a mixture of the labeled diphosphate **14** and glucoside **15** as substrates. The liberated isotopomers of citronellol showed a baseline separation in GC due to the high inverse isotope effect and could be analyzed by GC without MS detection. As a result of the cleavage experiments β-glucosidase showed a strong phosphatase side activity and phosphatase showed a strong β-glucosidase side activity. The β-glucosidase side activity of phosphatase could be inhibited by adding EDTA and gluconic acid δ-lactone to the buffer (Schwab, 1989). When the phloem exudate was incubated with β-glucosidase and inhibited phosphatase, respectively, only β-glucosidase liberated citronellol, which was unequivocally identified by MDGC-MS. This shows that glucosidically bound citronellol is translocated in *P. graveolens.* Phosphorylated citronellol could not be detected, despite the fact that citronellyl diphosphate is more effectively converted into rose oxide than the corresponding glucoside. Chubinidze et al. (1993) showed that radiolabeled citronellol, which was injected into stems of *P. roseum*, gives rise to products which are actively transported into leaves and roots. On the basis of the results of their study they conclude that citronellol is an active participant of metabolism in geranium cells and the products of its transformations are transported both upward and downward. The glucosidically bound citronellol that was identified in our study may act as a transport derivative and may be involved in metabolism as stated by Chubinidze et al.

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