Biogenesis of Geranium Oil Compounds: On the Origin of Oxygen in *cis*-/*trans*-Rose Oxide

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By feeding experiments with labeled [1,1-$^2H_2$,^{18}O]citronellol, it could be shown that *Pelargonium graveolens* is able to convert the fed precursor into *cis*-/*trans*-rose oxide. The labeling pattern and diastereomeric ratio of the resulting *cis*-/*trans*-rose oxides are in agreement with a mechanism which includes the enzymatic oxidation of citronellol in the allylic position and subsequent cyclization of the resulting diol to *cis*-/*trans*-rose oxide with retention of the oxygen of [1,1-$^2H_2$,^{18}O]citronellol.

Keywords: *Geranium oil; Pelargonium graveolens; rose oxide; solid-phase microextraction (SPME); stable isotope labeling*

INTRODUCTION

Geranium oil is one of the most important natural raw materials in the fragrance industry for creating rosy notes. It is also used as a flavoring material in, e.g., beverages, candies, and baked goods in varying concentrations (Duke et al., 1994). The essential oil is obtained by steam distillation of leaves and branches of various *Pelargonium* species and artificial hybrids. A minor but important olfactive ingredient of the essential oil is the so-called rose oxide (*cis*-/*trans*-2-(2-methyl-1-propenyl)-4-methyltetrahydropyran, **1**−**4**; see Scheme 1).

It was isolated from geranium oil by Naves et al. (1961). ^{1}H-NMR spectroscopic studies have revealed rose oxide as a 3/1 mixture of *cis*/*trans* diastereomers (Melera and Naves, 1961). The enantiomeric ratio of *cis*- and *trans*-rose oxide in various *Pelargonium* species was determined by enantio-MDGC and found to correlate with the enantiomeric ratio of citronellol, which is a main compound of the essential oil (Kreis and Mosandl, 1993). The biogenetic relation between rose oxide and citronellol in *Pelargonium* species was recently investigated by feeding experiments with labeled citronellyl glucosides (Wüst et al., 1996). It was shown that the plants are able to convert the fed citronellyl glucosides into the corresponding rose oxides. The mechanism of rose oxide formation has been investigated, pointing to an enzymatic oxidation rather than a photooxygenation of citronellol with subsequent cyclization of the resulting diol (Wüst et al., 1998). In this paper, we investigate the mechanistic aspects of rose oxide formation in more detail using mixed specifically labeled precursors.

EXPERIMENTAL PROCEDURES

Plant Material. Young plants of *Pelargonium graveolens* L'Héritier were kindly provided by Gartenbau Stegmeier, Essingen, Germany.

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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.

^{1}H-NMR. The spectra were recorded on a Bruker AMX 500; $CDCl_3$/TMS. NMR assignments were clarified by ^{1}H/^{1}H-COSY.

Enantio-MDGC−MS. The enantio-MDGC−MS (enantio-multidimensional gas chromatography−mass spectrometry) analysis of the SPME (solid-phase microextraction) 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, using an open split interface. Precolumn conditions: 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), then 2 °C/min to 250 °C. Main column conditions: 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 (polydimethylsiloxane), column was self-prepared according to Dietrich et al. (1995); carrier gas hydrogen 100 kPa; oven temperature 60 °C (25 min isothermal), then 2 °C/min to 180 °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 synthetic products were analyzed in the full scan mode (40−250 amu). The genuine and deuterium-labeled rose oxides **1**−**12** were detected in the SIM-mode (selected ion monitoring): *m*/*z* = 139−143. Live-T-cuttings (min): *cis*-/-*trans*-rose oxides (**1**−**12**) 10.00−12.00, split injection mode. Order of elution on main column: *cis*-rose oxide **1,5,9** (2*R*,4*S*), **2,6,10** (2*S*,4*R*); *trans*-rose oxide **3,7,-11** (2*R*,4*R*), **4,8,12** (2*S*,4*S*).

GC−MS. The GC−MS analysis of the synthetic products and the reference compounds was performed with a Fisions Instruments GC 8065, coupled to a Fisions Instruments MD 800 mass spectrometer, equipped with a HTFS capillary column (30 m × 0.25 mm; coated with SE-52; film thickness 0.5 μm; column was self-prepared according to Grob (1986)). GC conditions: carrier gas helium 70 kPa; split 30 mL/min; injector temperature 230 °C; oven temperature 40 °C (5 min isothermal), then 2.5 °C/min to 250 °C (30 min isothermal); ion source temperature 200 °C; mass range 40−250 amu; electron energy 70 eV. The molecular ions (M^+) and fragment ions are given as *m*/*z* with relative peak intensities in percent of the most abundant peak.

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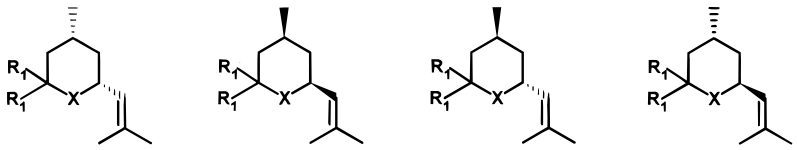
Scheme 1. Stereoisomers of Unlabeled and Labeled Rose Oxide



	cis-(2R,4S)	cis-(2S,4R)	trans-(2R,4R)	trans-(2S,4S)
R_1 = H, X = O	1	2	3	4
R_1 = D, X = O	5	6	7	8
R_1 = D, X = ^{18}O	9	10	11	12

Synthesis of 3,7-Dimethyl-[1,1-$^2H_2$,^{18}O]oct-6-en-1-ol ([1,1-$^2H_2$,^{18}O]Citronellol) (15). Sodium [^{18}O]hydroxide solution was prepared from sodium mercury amalgam beads (5% Na, 1.6 g, 3.5 mmol of Na) and $H_2{}^{18}O$ (96.7% atom ^{18}O, 0.93% atom ^{17}O, 2.37% atom ^{16}O; Euriso-top; Gif-sur-Yvette, France; 600 μL) according to Murray and Williams (1958). Three milliliters of dry ethanol and 0.7 mmol of citronellic acid nitrile **13** (Dragoco, Holzminden, Germany) were added, and the mixture was refluxed for 24 h with exclusion of moisture. The solvent was removed under reduced pressure, and the residue was acidified with 25% aqueous sulfuric acid. It was extracted 3 times with portions of 20 mL of ether, and the combined extracts were dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure to give 0.57 mmol of the crude acid **14** which was reduced without further purification: **14** was dissolved in 1 mL of dry ether and added carefully to a cooled suspension of 1.1 mmol of $LiAlD_4$ (>99% atom D; Fluka, Deisenhofen, Germany) in 1 mL of dry ether. It was refluxed for 1 h with exclusion of moisture and carefully quenched with water, and the precipitate was dissolved by adding 25% aqueous sulfuric acid. It was extracted 3 times with portions of 20 mL of ether, and the combined extracts were dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure, and 20 mg of the crude product was purified by preparative TLC (precoated PLC plates, silica gel 60 F-254 (Merck, Darmstadt, Germany), eluent pentane/ether 7/3 (v/v)) to give 14 mg (0.1 mmol) of pure product with an enantiomeric ratio (*S*)/(*R*) of 42/58 (enantioselective GC): ^{1}H-NMR identical with the data given by Wüst et al. (1998); MS 160 (M^+, 2), 140 (7), 125 (13), 109 (9), 97 (21), 83 (46), 69 (100), 55 (34), 41 (88). For deuterium and ^{18}O contents, see corresponding rose oxides **9**−**12**.

Synthesis of *cis*-/*trans*-2-(2-Methyl-1-propenyl)-4-methyl-[6,6-$^2H_2$,^{18}O]tetrahydropyran (*cis*-/*trans*-[6,6-$^2H_2$,^{18}O]-Rose oxide) (9−12). 9−**12** were prepared according to Taneja et al. (1978) with some modifications. Ten milligrams of crude **15** and a catalytic amount of *N*-iodosuccinimide (NIS) were taken up in 0.5 mL of CCl_4 in a GC vial. The vial was sealed and heated at 95 °C for 20 min. The dark violet solution was shaken several times with an aqueous solution of $Na_2S_2O_3$ until the iodine was completely removed; 250 μL of the reaction mixture was subjected to preparative TLC (Polygram SIL G/UV$_{254}$, Machery-Nagel, Düren, Germany; eluent: pentane/ether 1/1 (v/v)). The rose oxide fraction was removed from the TLC plate and extracted with 0.5 mL of ether. The *cis*/*trans* ratio of **9**/**12** and **10**/**11** was found to be 63/37 (GC−MS). The enantiomeric ratio of **9**/**10** and **11**/**12** was found to be 57/43 (enantio-MDGC−MS): MS *cis*-**9**/**10** 158 (M^+, 9), 143 (100), 141 (8), 139 (0.2), 123 (2), 116 (4), 101 (8), 85 (52), 71 (72), 67 (14), 55 (25), 41 (25); *trans*-**11**/**12** 158 (M^+, 7), 143 (100), 141 (8), 139 (0.2), 123 (3), 116 (5), 101 (9), 85 (57), 71 (76), 67 (16), 55 (26), 41 (31); deuterium and ^{18}O contents [$^2H_2$,^{18}O] >92%, [$^2H_2$,$^{18}O_0$] <8%, [$^2H_0$,$^{18}O_0$] <1%.

Synthesis of *cis*-/*trans*-2-(2-Methyl-1-propenyl)-4-methyl-[6,6-$^2H_2$]tetrahydropyran (*cis*-/*trans*-[6,6-$^2H_2$]Rose oxide) (5−8). 5−**8** were prepared according to Wüst et al. (1998).

Administration of the Labeled Precursor. A solution of **15** with a concentration of 0.1 mg/mL (625 μM) was prepared by dissolving the same amounts of **15** and Tween 20 in water. Then 100 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.

SPME. A 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 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.

The splitless injection mode was used, the split valve being opened after 2 min.

RESULTS AND DISCUSSION

To investigate the cyclization of citronellol to rose oxide in *Pelargonium* species in more detail, the mixed specifically labeled [1,1-$^2H_2$,^{18}O]citronellol **15** was synthesized according to Scheme 2. Labeled reference compounds **5**−**12** were synthesized by cyclization of **15** and **17** using NIS (Taneja et al., 1978). A comparison of the mass spectra of unlabeled genuine *cis*-rose oxides **1**, **2** and labeled *cis*-rose oxides **9**, **10** and **5**, **6** (see Figure 1) shows that the genuine rose oxides can be detected selectively on mass lane 139, the deuterium-labeled rose oxides **5**−**8** on mass lane 141, and the mixed-labeled rose oxides **9**−**12** on mass lane 143.

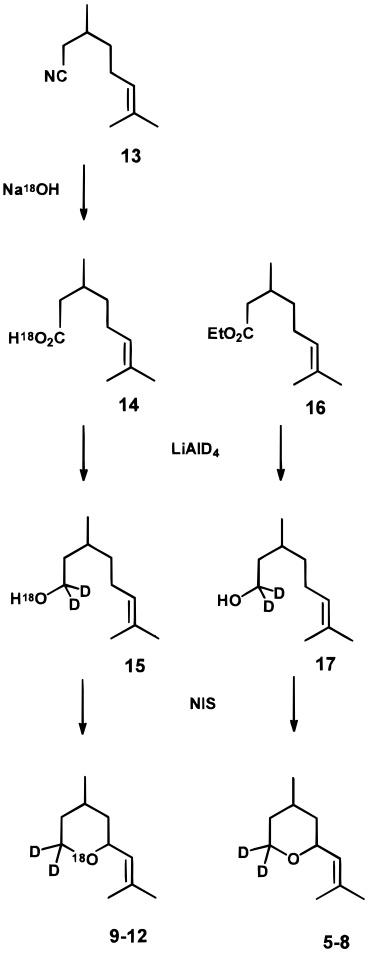
The mixed specifically labeled [1,1-$^2H_2$,^{18}O]citronellol **15** was fed to the plant by incubating leaf disks with the feeding solution in septum-sealed 2 mL vials for 24 h in the dark. These conditions were chosen in order to exclude a photooxygenation of labeled citronellol **15**. After the incubation, the headspace of the septum-sealed vial was analyzed using solid-phase microextraction (SPME). The SPME extracts were analyzed by enantio-MDGC−MS. Figure 2 shows a precolumn chromatogram obtained from *P. graveolens* which was fed with **15**. The *cis*-/*trans*-rose oxides were transferred to the chiral main cloumn and detected by MS. Figure 3 shows the main column chromatogram detected in the SIM mode (m/z = 139−141).

Mass lane 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**) configurated *cis*-/*trans*-rose oxides is approximately 3/1 (see Table 1). Mass lane 143 shows the presence of mixed specifically labeled *cis*-/*trans*-[6,6-$^2H_2$,^{18}O]rose oxide **9**−**12**. This fact proves that the cyclization of [1,1-$^2H_2$,^{18}O]citronellol **15** to rose oxide proceeds with reten-

Table 1. Enantiomeric and Diastereomeric Ratios of Genuine and Labeled Rose Oxides from *Pelargonium graveolens* Fed with 15 (Standard Deviation in Parentheses; $N = 4$)

	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 (0.1)	98/2 (0.9)	76/24 (0.8)	76/24 (5.4)
143 (labeled)	55/45 (1.7)	43/57 (1.9)	76/24 (1.0)	74/26 (1.9)

Scheme 2. Synthesis of Labeled Citronellol and Reference Compounds



tion of the oxygen of citronellol. If the cyclization of [1,1-$^2H_2$,^{18}O]citronellol **15** had proceeded by loss of the oxygen atom, the resulting *cis*-/*trans*-[6,6-$^2H_2$]rose oxide should have given a signal on mass lane 141. But on mass lane 141 only trace amounts of *cis*-/*trans*-[6,6-$^2H_2$]-rose oxide are detected which are due to the small content of [1,1-$^2H_2$]citronellol in the feeding substance (see Experimental Procedures; the $H_2{}^{18}O$ which was used for the synthesis of **15** has approximately 2.4% atom residual ^{16}O). The labeled *cis*- and *trans*-[6,6-$^2H_2$,^{18}O]rose oxides **9**−**12** show almost the same enantiomeric ratio as the precursor **15** itself (see Table 1; *R*/*S* of **15** 58/42; the starting material of the synthesis of **15** was citronellic acid nitrile **13** obtained from Dragoco (Holzminden, Germany) which was obviously not racemic; probably it was prepared from natural sources). This shows that the cyclization of citronellol in the studied plant is a rather unspecific process without any detectable discrimination between the enantiomers of rose oxide (Wüst et al., 1996).

The fact that the cyclization of citronellol also proceeds in the dark excludes a photooxygenation mechanism which was advanced by Ohloff et al. (1961, 1975) in previous biomimetic studies. In these studies, it was

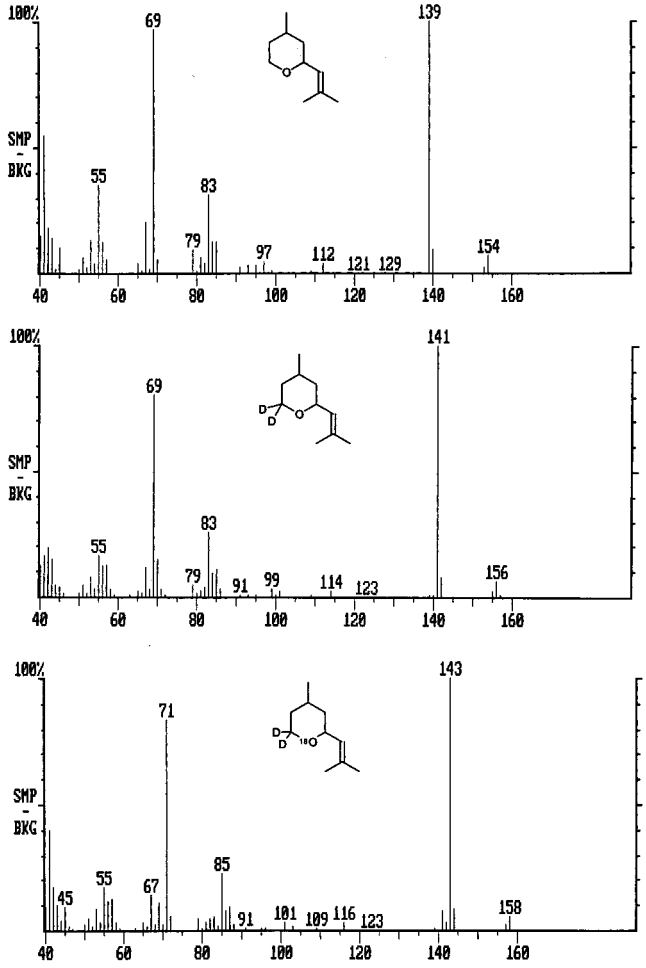


Figure 1. Mass spectra of unlabeled and labeled *cis*-rose oxides.

presumed that the biogenetic route of rose oxide formation must be very similar to the synthetic route via photooxygenation of citronellol to give the diol **18** which can cyclize to *cis*-/*trans*-rose oxide by acid-catalyzed elimination of water. Chlorophyll was discussed as a suitable sensitizer for the formation of singlet oxygen in the plant. Since a photooxygenation mechanism can be excluded, it is highly probable that citronellol is enzymatically oxydized in the allylic position to give one of the diols in Scheme 3 (**18** or **19**) which are equivalent by allylic rearrangement. **18**/**19** can easily cyclize to rose oxide as discribed above.

The diastereomeric ratio of *cis*- and *trans*-rose oxides which are generated by cyclization of **18**/**19** is 3/1 (Patel et al., 1977; Snowden et al., 1987) and is identical with the genuine *cis*/*trans* ratio in *Pelargonium* plants. The labeled rose oxides **9**−**12** resulting from the fed precursor **15** also show a *cis*/*trans* ratio of 3/1 (see Table 1). The acid-catalyzed cyclization of the diols **18**/**19** implies retention of the nonallylic hydroxy group and leaving of the activated allylic hydroxy group (see Scheme 3). This is in agreement with the labeling pattern of the rose oxides resulting from the cyclization of the fed

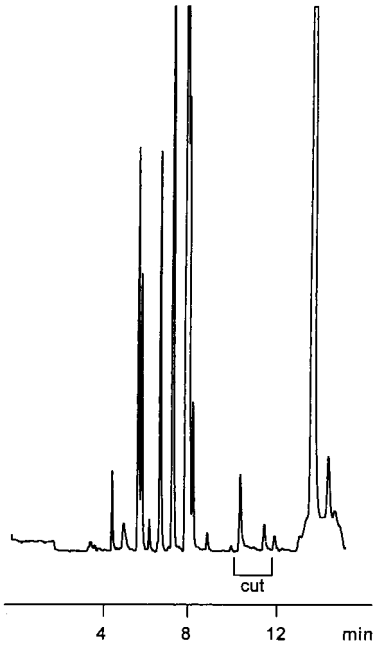


Figure 2. Precolumn chromatogram of a SPME headspace extract of *Pelargonium graveolens* fed with **15**.

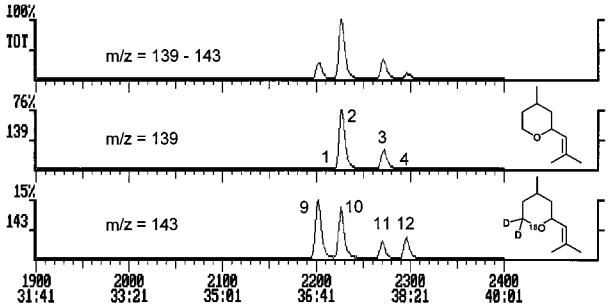
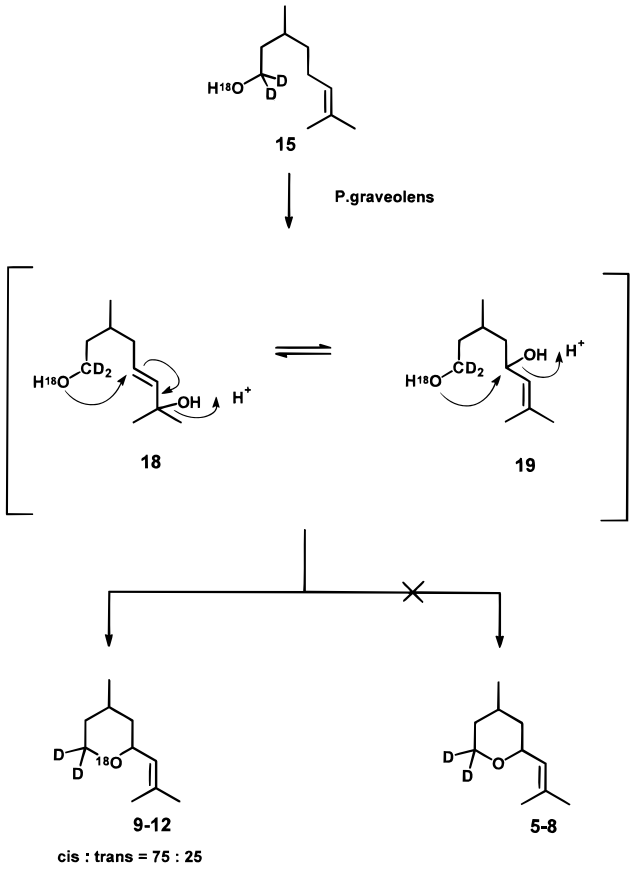


Figure 3. Main column chromatogram of a SPME headspace extract of *Pelargonium graveolens* fed with **15**; detected in SIM mode (m/z = 139−142).

precursor **15**. The loss of the nonallylic hydroxy group can only be initiated by activation of this hydroxy group. Chemically this can be done by selective tosylation of the nonallylic hydroxy group (Fronza et al., 1992). Obviously this mechanism does not fit the experimantal data and can, hence, be excluded. Due to the low pH value of the *Pelargonium* plant tissue (approximately 3.5), the diols **18**/**19** could not be isolated up to now. Presumably the cyclization of **18** or **19** takes place immediatly after the enzymatic oxidation of citronellol. In this context it should be mentioned that in petals of *Rosa damascena* the diol **18** was recently isolated and spectroscopically characterized (Straubinger et al., 1998). Additionally the vacuum headspace distillation extract of rose petals contains no rose oxides (Surburg et al., 1993). These facts show that in *Rosa damascena* the diol **18** is the precursor of rose oxide (Straubinger et al., 1998). Obviously the cyclization of the diol **18** is blocked in intact rose petals and is initiated by steam distillation to obtain the essential oil. In contrast, in headspace extracts of intact *Pelargonium* plants, *cis*-/*trans*-rose oxide can be detected (Wüst et al., 1996, 1998). Furthermore, it is interesting to note that rose oxide can be degraded to the so-called rose oxide ketone in *Pelargonium* plants as could be shown by enantio-MDGC−MS analysis (Wüst et al., 1997).

Scheme 3. Formation of Rose Oxide in *Pelargonium graveolens*



CONCLUSIONS

By feeding the mixed specifically labeled precursor citronellol **15**, it could be shown that *Pelargonium graveolens* is able to convert the fed precursor into *cis*-/*trans*-rose oxide. Since it was incubated in the dark, a photooxygenation mechanism could be excluded. The labeling pattern and *cis*/*trans* ratio of the resulting rose oxides are in agreement with a mechanism which includes the enzymatic oxidation of citronellol in an allylic position and subsequent cyclization of the resulting diol to *cis*-/*trans*-rose oxide. These findings may be of fundamental interest in IRMS measurements of $^{16}O/^{18}O$ of citronellol and rose oxide for authenticity assessments of geranium oils.

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