210. Regioselectivity and Deuterium Isotope Effects in Geraniol Hydroxylation by the Cytochrome P-450 Monooxygenase from *Catharanthus roseus* (L.) G. DON

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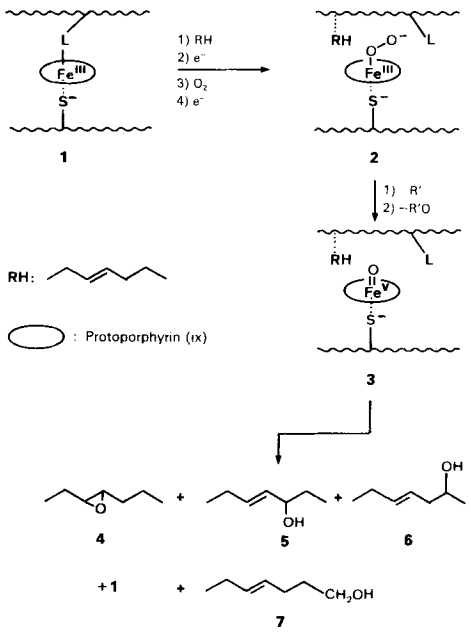
The hydroxylation of geraniol (**8**) by cytochrome P-450 (P-450$_{Cath.}$) from the subtropical plant *Catharanthus roseus* (L.) G. DON was optimised to give 8-hydroxygeraniol (**9**) as the single product in 35% yield. Incubations of different ^{13}C- and ^{2}H-labelled geraniols revealed that H-abstraction is completely regioselective in favour of the CH_3 group *trans* to the chain at C(6) of **8**. An intramolecular isotope effect $k_H/k_D = 8.0$ was determined, suggesting that H-abstraction is one of the major rate-contributing steps; however, the intermolecular isotope effect was surprisingly inverse at low conversion $k_H/k_D = 0.50$, indicating the existence of rate-contributing steps preceding the first irreversible, isotope-sensitive reaction in the sequence.

Introduction. – Cytochrome-P-450-dependent monooxygenases are an ubiquitious class of enzymes which have in common an iron protoporphyrin(IX) substructure in order to bind and activate molecular oxygen (see **1** and **2**) [1–3]. At present, it is accepted that the bound oxygen is reductively cleaved generating an iron-oxene species (see **3**),

Scheme 1



being able to transfer its O-atom to the substrate to yield alcohols, epoxides, and allylic alcohols (see **4**–**7**; *Scheme 1*). Due to these unique features, these enzymes play a key role in the metabolism of endogenous compounds and xenobiotics.

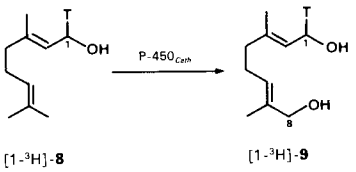
Although considerable knowledge has been accumulated in recent years from comparison of the enzymes with synthetic model compounds, the mechanisms of O_2-cleavage and O-transfer are still in debate [4–6]. Concerning the stereoselectivity of substrate oxidation, several types of reactions have been investigated in more detail: the hydroxylation of non-activated CH_2 groups [7–10], the hydroxylation of non-activated CH_3 groups [10] [11], and the hydroxylation of benzylic CH_2 groups [12–14]. Although not always established with absolute certainty, these investigations led to the general conclusion that P-450-catalysed hydroxylations proceed under retention of configuration. This unified picture of a concerted insertion of oxygen into a C–H bond was questioned recently by experiments involving the hydroxylation of certain cyclic substrates. It was found by *Sligar* and coworkers [15] that, although the hydroxylation of camphor by P-450$_{cam}$ is overall stereo- and regioselective yielding 5-*exo*-hydroxycamphor, the H-abstraction is unselective. Stereochemical scrambling was also observed by *Groves* and *McClusky* during the hydroxylation of norbornane [16] and regiochemical scrambling established in the hydroxylation of endo- and exocyclic olefins [17]. These results support the formation of a carbon radical by H· abstraction; the stereo- and regioselectivity of the process is then depending on the lifetime of C· and the ratio rate of recombination/rate of rearrangement, respectively.

The kinetic isotope effects calculated for P-450-catalysed hydroxylations are in most cases intermolecular ($k_H/k_D < 2$) [10] [14] [18], accounting for the contribution of the rate-determining steps other than H-abstraction to the total rate. According to *Northrop* [19], such low, apparent, intermolecular isotope effects are difficult to interprete in terms of identifying the C–H bond cleavage step as rate-limiting. Intramolecular isotope effects have been calculated in only a few cases. *Groves* and *McClusky* calculated $k_H/k_D = 11.5$ for the hydroxylation of norbornane [16], and *Hjelmeland et al.* estimated $k_H/k_D = 6$–11 for H-abstraction from 1,3-diphenylpropane [13]. Recently, careful investigations of the P-450-catalysed dealkylation of 7-ethoxycoumarin revealed a significant intramolecular isotope effect ($k_H/k_D = 5.5$; equal to the calculated intrinsic value) with hamster liver microsomes [20] and very high intrinsic isotope effects ($k_H/k_D = 12.8$–14.0) with purified rat liver isozymes [21].

To the best of our knowledge, the cytochrome-P-450-catalysed hydroxylation of allylic CH_3 groups has not been investigated with respect to regio- and stereoselectivity, and intra- and intermolecular isotope effects. In view of the results discussed above, this case is of particular mechanistical interest, since H· abstraction is anticipated to give an allylic radical which is considerably stabilised. Furthermore, both inter- and intramolecular isotope effects can be measured without interference by enantioselective H· abstraction.

Our initial aim to tackle this problem was to select an enzymatic system which would allow the investigation of the substrate and the products by NMR in order to avoid difficult MS analysis. The system of choice was the cytochrome-P-450-dependent monooxygenase from the readily available, indole-alkaloids-containing, subtropical plant *Catharanthus roseus* (L.) G. DON [22], which is reported to catalyse the oxidation of geraniol (**8**) to 8-hydroxygeraniol (**9**; *Scheme 2*). This reaction has been investigated by *Meehan*

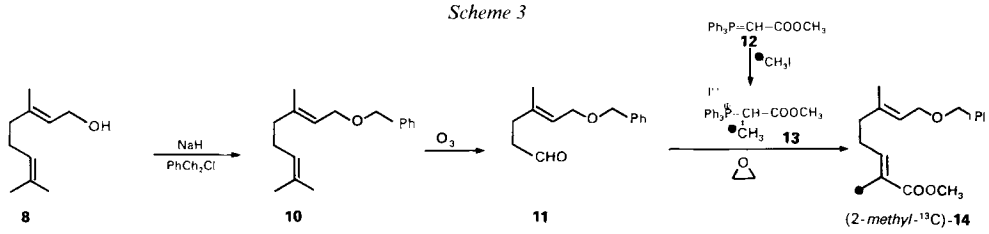
Scheme 2



and *Coscia* [23] with unpurified cytochrome P-450$_{Cath.}$ in a cell-free system of *C. roseus*, and a maximal yield of 5% for **9** has been achieved under various isolation and incubation conditions [24] [25]. However, we realised that no unequivocal evidence existed for **9** as the product of this oxidation, since **9** has never been isolated 'neat' from any incubation, but only by addition of a $\geq$ 1000-fold excess of inactive **9** to the radioactive oxidation mixture. The only indication that **9** would be an intermediate between **8** and the terpenoid moiety of the indole alkaloids stems from the incorporation of labelled **9** into loganin and the alkaloids [26] [27].

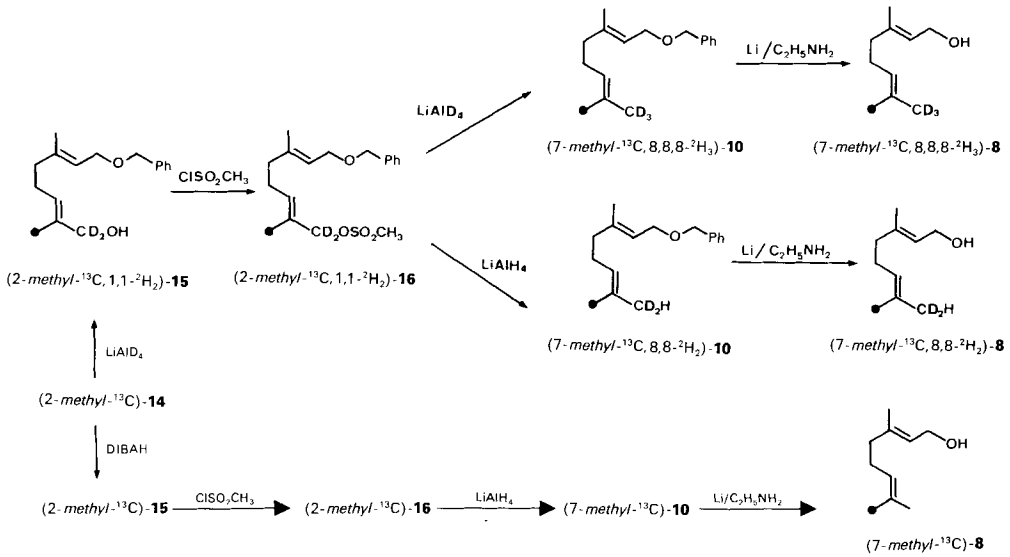
Results and Discussion. – To investigate the monooxygenase system from *Catharanthus roseus* (L.) G. DON, the synthesis of various ^{13}C- and ^{2}H-labelled geraniols carried out is outlined in *Schemes 3–5*.

Scheme 3



The terminal double bond of geranyl benzyl ether **10** was selectively cleaved by O_3 [28] and the resulting aldehyde **11** subjected to a modified *Wittig* reaction [29] by treatment with the ^{13}C-labelled phosphonium salt **13**, which was obtained from $^{13}CH_3I$ and **12** [30] *(Scheme 3)*. The unsaturated ester (2-*methyl*-^{13}C)-**14** was obtained in 43% from $^{13}CH_3I$ and was shown, by GLC, and ^{1}H- and ^{13}C-NMR, to contain 98% of the (2*Z*)-isomer and 2% of the (2*E*)-isomer. The mixture of isomers was reduced with diisobutylaluminium hydride (DIBAH) or $LiAlD_4$ to give the alcohols (2-*methyl*-^{13}C)-**15** and (2-*methyl*-^{13}C, 1,1-$^2H_2$)-**15** in 97% and 70% yield, respectively *(Scheme 4)*. Subsequent mesylation furnished the esters (2-*methyl*-^{13}C)-**16** and (2-*methyl*-^{13}C, 1,1-$^2H_2$)-**16**, respectively, which were not characterised but immediately reduced with $LiAlH_4$ or $LiAlD_4$ to the olefins (7-*methyl*-^{13}C)-**10**, (7-*methyl*-^{13}C, 8,8-$^2H_2$)-**10**, and (7-*methyl*-^{13}C, 8,8,8-$^2H_3$)-**10** [31]. Ether cleavage by Li in $EtNH_2$ gave the geraniols (7-*methyl*-^{13}C)-**8**, (7-*methyl*-^{13}C, 8,8-$^2H_2$)-**8**, and (7-*methyl*-^{13}C, 8,8,8-$^2H_3$)-**8**. All three compounds were pure by TLC and GLC (> 95%). According to ^{13}C- and ^{1}H-NMR, the ^{13}C-label was located in the CH_3 group (17.6 ppm) *cis* to the chain at C(6) of **8** to > 98%. The ^{13}C-enrichement was 90% (^{1}H-NMR) and 95% (MS) in agreement with the specification by *Merck-Sharp & Dohme*. According to ^{1}H-NMR, the ^{2}H enrichement was 100% and, by MS, measured to be

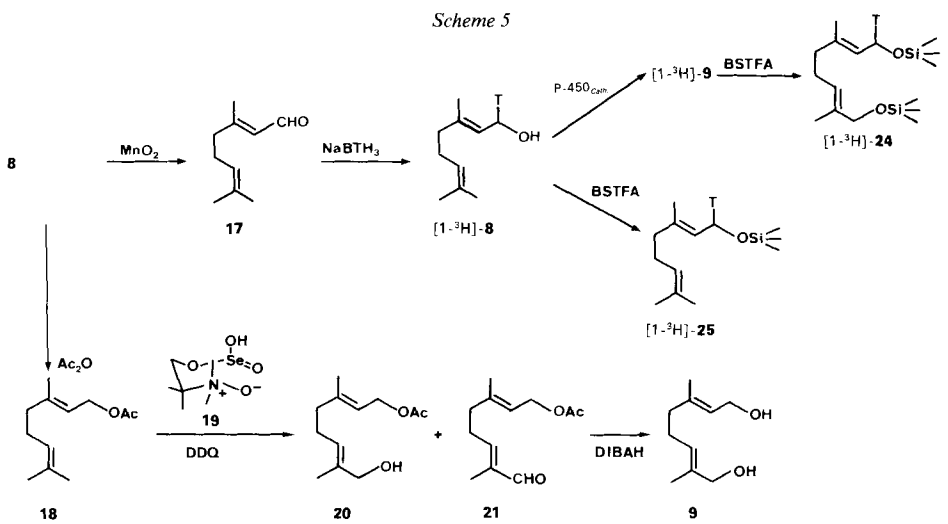
Scheme 4



greater than 98% at the desired positions. The tritium-labelled geraniol [1-^{3}H]-**8** was prepared from **8**, *via* **17**, by standard methods *(Scheme 5)*. This compound, having a specific activity of 138 mCi/mmol and being pure (GLC and radio-TLC), was used to establish optimal incubation conditions for the conversion of **8** to **9**.

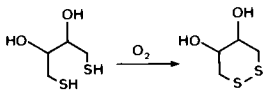
A reference sample of 8-hydroxygeraniol (**9**) was prepared by allylic oxidation of geranyl acetate (**18**) and subsequent reduction of the products **20** and **21** (see *Scheme 5*), using a new, high-yielding transformation [32] which involves the application of the [(selenino)oxy]ethylamine oxide **19** and the co-oxidant 4,5-dichloro-3,6-dioxo-1,4-cyclohexadiene-1,2-dicarbonitrile (DDQ).

Scheme 5



The monooxygenase was isolated from 5-days-old colourless seedlings of *C. roseus* and identified by its CO-difference spectrum [24] [33]. During our attempts to isolate [1-^{3}H]-**9** 'neat' under reported incubation and isolation conditions [23], we faced the problem never mentioned by *Coscia* and coworkers [23] [24] that, in many silica-using TLC-systems including *Coscia*'s [23], [1-^{3}H]-**9** was contaminated by large amounts of the cyclic disulfide **23** *(Scheme 6)*, identified by NMR and MS comparison with a synthetic sample. Disulfide **23** is formed from the incubation additive dithiothreitol (DTT; **22**) which is required in order to prevent the oxidation of cysteine moieties of the enzyme [34].

Scheme 6



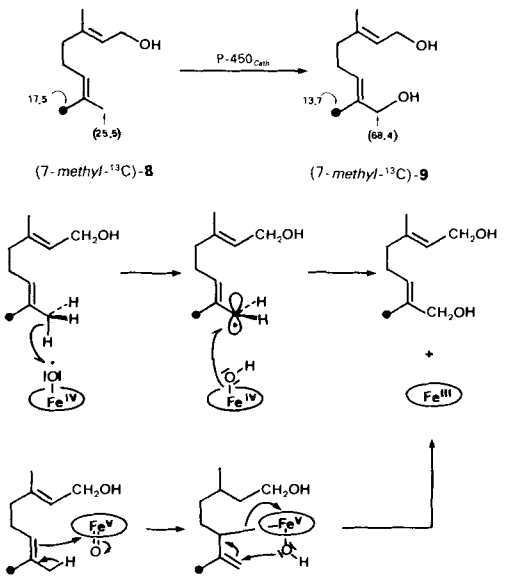
22 **23**

Omitting the additive **22** from the incubation mixture resulted in considerable loss of monooxygenase activity. However, enough [1-^{3}H]-**9** was formed so that, after silylation of the whole extract with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), a direct comparison of the trimethylsilyl derivative [1-^{3}H]-**24** (see *Scheme 5*) was possible with the corresponding derivative **24** of non-radioactive **9** using combined MS(EI)/GLC (radioactivity detector). The radio-GLC of the silylation mixture showed only two radioactive peaks, one being identical with the trimethylsilyl derivative [1-^{3}H]-**25** of [1-^{3}H]-**8** (see *Scheme 5*) and the other arising from [1-^{3}H]-**24**, with the same retention time as the co-injected inactive **24**; the MS of the peak of [1-^{3}H]-**24** was identical with the reference MS of **24**. Accordingly, it is now unequivocally established that 8-hydroxygeraniol (**9**) is the only product of the hydroxylation of geraniol (**8**) catalysed by cytochrome P-450 from *C. roseus*.

Since the problem of separating disulfide **23** from **9** was eventually solved by chromatography on Al_2O_3, we attempted to find optimal conditions for the incubation of **8** in order to analyse hydroxylations by ^{1}H- and ^{13}C-NMR. Best results were achieved in the following way (see *Exper. Part*): Unpurified protein was suspended in 0.1M phosphate buffer (pH = 7.2) in the presence of a 7- to 8-fold excess of NADPH and of dithiothreitol (DTT; **22**). The reaction was started by adding [1-^{3}H]-**8** in acetone. After 30 min stirring under aerobic conditions, the reaction was quenched by addition of MeOH and extracted for the terpenes. A reproducible 35% conversion (max. 47%) for [1-^{3}H]-**8**→[1-^{3}H]-**9** was detected by radio-TLC scanning, accounting for a monooxygenase activity of 1 nmol substrate/min·mg protein. Within an error of 2%, no radioactive compounds other than [1-^{3}H]-**8** and [1-^{3}H]-**9** were present, excluding the possible formation of aldehydes and acids; this has been also confirmed by GLC and is an important fact concerning isotope effects *(vide infra)*.

After incubating synthetic (7-*methyl*-^{13}C)geraniol ((7-*methyl*-^{13}C)-**8**) with P-450$_{Cath.}$ under standard conditions *(Scheme 7)*, the starting material was recovered spectroscopically unchanged, > 98% of the ^{13}C-label was still present in the CH_3 group *cis* to the chain at C(6). The GLC-pure product 8-hydroxy(7-*methyl*-^{13}C)geraniol ((7-*methyl*-^{13}C)-**9**) displayed only one ^{13}C-NMR signal at 13.7 ppm and no ^{13}C-enrichment for the signal at 68.4 ppm (C(8) of **9**). Therefore, in contrast to the SeO_2 oxidation [35] [31], the chemical

Scheme 7

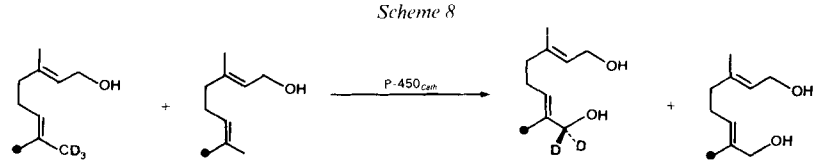


(7-*methyl*-^{13}C)-**8** (7-*methyl*-^{13}C)-**9**

equivalent of this enzymatic process, the P-450$_{Cath.}$-catalysed hydroxylation of geraniol proceeds with complete regioselectivity. However, the incubation experiment does not distinguish between two mechanistical alternatives: an insertion of the O-atom into a C–H bond of the CH_3 group *trans* to the chain at C(6) of **8** and a regio- and stereoselective two-step sequence including an ene reaction followed by a [2,3]-sigmatropic rearrangement *(Scheme 7)*. The latter mechanism would involve the formation of an intermediate having the substrate covalently bound to the Fe atom of the P-450$_{Cath.}$.

To determine the intermolecular isotope effect concerning H-abstraction from the CH_3 group *trans* to the chain at C(6) of **8**, a 1:1 mixture of (7-*methyl*-^{13}C)-**8** and (7-*methyl*-^{13}C,8,8,8-$^2H_3$)-**8** was incubated under standard conditions *(Scheme 8)*, and the incubation quenched after 5, 10, 15, and 30 min. The resulting mixtures of (7-*methyl*-^{13}C)-**9** and (7-*methyl*-^{13}C,8,8-$^2H_2$)-**9** were thoroughly purified and subjected to ^{1}H-NMR analysis. The ratio of the deuterated to the undeuterated hydroxygeraniol was determined by comparison of the integration of the resonance for the protons at C(8) (*d* at 4.01 ppm) with that of the signal for the protons at C(1) (*d* at 4.18 ppm). From these data, it was calculated that the time-dependent intermolecular isotope effect is surprisingly inverse at low conversion, $(k_H/k_D)_{inter} = 0.50$ (5 min), 0.75 (10 min), 0.87 (15 min), 1.02 (30

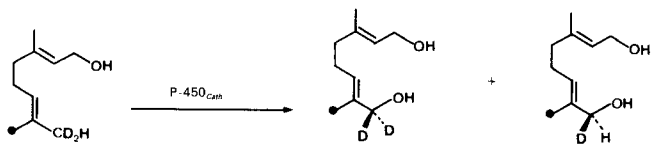
Scheme 8



(7-*methyl*-^{13}C,8,8,8-$^2H_3$)-**8** (7-*methyl*-^{13}C)-**8** (7-*methyl*-^{13}C,8,8-$^2H_2$)-**9** (7-*methyl*-^{13}C)-**9**

min). These data do not indicate artefact formation due to overoxidation of 8-hydroxygeraniol (**9**), as demonstrated by the following control experiment. Incubation of a 1:1 mixture of (7-*methyl*-^{13}C)-**9** and (7-*methyl*-^{13}C,8,8-$^{2}H_2$)-**9**, containing [1-^{3}H]-**9**, under standard conditions for 30 min resulted in tritium-labelled 8-hydroxygeraniol, being pure by TLC (radioactivity scanning); synthetic samples of all possible oxidation products were clearly separated from [1-^{3}H]-**9**. Workup of the incubation mixture revealed 1.4% of the total radioactivity present in the lyophylised H_2O phase, and 98.6% in the organic layer from which hydroxygeraniol was isolated, displaying an unchanged ratio of 1:1 for (7-*methyl*-^{13}C)-**9**/(7-*methyl*-^{13}C,8,8-$^{2}H_2$)-**9** in the ^{1}H-NMR spectrum.

Scheme 9



(7-*methyl*-^{13}C,8,8-$^{2}H_2$)-**8** (7-*methyl*-^{13}C,8,8-$^{2}H_2$)-**9** (7-methyl-^{13}C,8-^{2}H)-**9**

The intramolecular isotope effect was investigated by incubation of (7-*methyl*-^{13}C,8,8-$^{2}H_2$)-**8** with cytochrome P-450$_{Cath.}$ under standard conditions (*Scheme 9*). By comparison of the ^{1}H-NMR signals at 4.01 and 4.18 ppm, the ratio of dideuterated (7-*methyl*-^{13}C,8,8-$^{2}H_2$)-**9** to monodeuterated (7-*methyl*-^{13}C,8-^{2}H)-**9** was calculated to be 4.0 accounting for $(k_H/k_D)_{intra} = 8.0 \pm 0.5$ (avarage of 4 experiments).

This discrimination between ^{1}H and ^{2}H in the intramolecular case is suggesting that H-abstraction is one of the major rate-contributing steps in the hydroxylation of geraniol (**8**). However, the intermolecular isotope effect in the regioselective hydroxylation of **8**, which is essentially an effect on V/K including all reactions up to the first irreversible, isotope-sensitive step (H-abstraction) is inverse at low conversion. This result is indicative of rate-contributing reactions preceding the C–H bond-cleavage step.

Two categories of such rate-contributing reactions could be envisaged in the hydroxylation of **8**: those which are directly substrate-related, such as the formation of the E·S complex involving the displacement of the sixth ligand by substrate and oxygen (see **1**→**2**, *Scheme 1*), the interaction between the substrate double bond and the Fe-atom (see *Scheme 7*), and those reactions which are substrate-independent and can be looked at as being decoupled from the product-formation steps, *e.g.* slow delivery of electrons by the P-450 reductase or irreversible formation of the active iron-oxene intermediate [21] (see *Scheme 1*).

To obtain a more precise picture of the allylic hydroxylation performed by this plant P-450 monooxygenase, work is in progress concerning stoichiometric, temperature-dependent studies and the determination of intrinsic isotope effects [19], to evaluate whether the isotope effects observed in the present study are fully expressed.

We thank *Renata Farner-Kamensky* for preliminary experiments, Dr. *G. Oesterhelt (F. Hoffmann-La Roche & Co., AG)* for GLC/MS-GLC/radioactivity detector measurements, Dr. *J. Schmid (Givaudan Forschungsgesellschaft AG)* for MS, and Dr. *A. Hafner* and *Umberto Piantini*, Universität Zürich, for NMR spectra. A generous sample of GLC pure geraniol was provided by *Givaudan Forschungsgesellschaft AG*. For generous financial support, we thank the *Swiss National Science Foundation* and *Givaudan Forschungsgesellschaft AG*, Dübendorf.

Experimental Part

General. $NaBH_3[^3H]$ was purchased from *Amersham*, Buckinghamshire, England, and $^{13}CH_3I$ from *Merck Sharp & Dohme*, Montreal, Canada. A GLC pure (97.5%) sample of geraniol (**8**) was received from *Givaudan Forschungsgesellschaft AG*, Dübendorf. THF and Et_2O were distilled from Na and benzophenone; $CHCl_3$ was used only after filtration through basic Al_2O_3 *(Woelm)*. If not otherwise stated, all reagents were of *'puriss.'* quality and purchased from *Fluka AG* (Buchs). For ozonolysis, a *Welsbach* ozon generator, mod. *502 (Fischer AG)*, was used. The cell-free extracts from *Catharanthus roseus* were prepared by centrifuging on a *TGA-65* ultracentrifuge *(Kontron)* having a titanium fixed angle rotor *TFT 65.38* (r_m 6.75 cm). Evaporations were performed under reduced pressure using a *Büchi* rotary evaporator and finally at 0.01 Torr using an *Alcatel* oil pump.

TLC: purity control on 5 × 7.5-cm aluminium sheets, SiO_2 *60* F_{254} (precoated; *Merck*); detection by UV_{254} light or spraying with $KMnO_4$ soln.; the radioactivity distribution was measured on a *Berthold-TLC* scanner *(II LB 2723)*; TLC after enzymatic reactions on 20 × 20-cm glass plates having 0.25-mm layers of SiO_2 *60* F_{254} (precoated; *Merck*) or Al_2O_3 *60* F_{254} (type *E*, precoated; *Merck*). Column chromatography (CC): SiO_2 *60* (0.063–0.200 mm, 70–230 mesh ASTM; *Merck*). GLC: purity control on capillary columns (25 m × 0.25 mm *BP5* or 25 m × 0.32 mm *SE54*). GLC radioactivity detection: *Packard* GC mod. *427* coupled with a *Packard* gas proportional counter mod. *894* (GLC: 20 m *SE54*, 5% *GC Q* 80/100 mesh, temp. programme 80–310° (6°/min), injector temp. 270°, detector temp. 300°, interface temp. 300°, FID 1 × 8; counter: CuO/Fe-catalysator oven, 780°, voltage 1750 V, range 20000 counts. GLC/MS: *Varian 2740/MAT CH7* (GLC: 20 m *SE54*, temp. programme 80–370° (8°/min), interface 280°). UV spectra: *Uvicon-810* spectrometer *(Kontron)*. ^1H- and ^{13}C-NMR spectra: *Varian XL-200* (^1H(200 MHz), ^{13}C(50 MHz)) or *Bruker AM-400* (^1H(400 MHz), ^{13}C(100.6 MHz)) spectrometer; $CDCl_3$ solns. if not otherwise stated; δ in ppm relative to TMS, coupling constants J in Hz. MS: *Varian-112S* spectrometer (EI: 70 eV; CI: isobutan).

Plant Material. Catharanthus roseus seeds (*Samen Mauser*, Dübendorf) were germinated in the dark at 32° on a layer of moist vermiculite for 5 days [36]. Etiolated seedlings were collected and the seed coats removed from the cotyledons.

Preparation of Microsomes. All subsequent operations were carried out at 0–4°. Seedlings (8 g) were ground to a fine powder under liq. N_2 and homogenised by 10 strokes of a hand-driven glass pestle in a glass homogeniser containing 40 ml of 0.2M potassium-phosphate buffer (pH = 7.2), containing 0.01M $Na_2S_2O_5$, 1 mM DTT (dithiothreitol), and 0.01M EDTA. The suspension was sonicated for 20 s in an ultrasonic bath, and after centrifugation at 12000*g* for 20 min, the supernate was sedimented at 110000*g* for 90 min. The microsomal fraction, received as a pellet [23] [36], was resuspended in 60 ml of 0.1M potassium-phosphate buffer (pH 7.2), containing 1 mM DTT and 1 mM EDTA to afford a final protein concentration of *ca.* 1 mg/ml as determined by the method of *Lowry* [37]. According to the procedure of *Sato* [33], cytochrome P-450$_{Cath.}$ was identified by its characteristic 450-nm CO-difference spectrum of the dithionite-reduced form.

Standard Incubation and Assay Conditions. If not otherwise stated, 60 mg of microsomal protein (110000*g* pellet) were incubated in the presence of 43 mg (0.05 mmol) of NADPH, 1 mM DTT, 1 mM EDTA, and 0.1M potassium-phosphate buffer (pH 7.2) in a total volume of 60 ml. Hydroxylation was initiated by addition of 1.0 mg (0.0065 mmol) of geraniol (**8**) in 0.5 ml of aceton, and the resulting soln. was gently stirred under aerobic conditions for 30 min at 25°. The reaction was quenched by addition of 60 ml of MeOH and extracted 3 times with 70 ml of $CHCl_3$. After evaporation of the solvents, substrate **8** and product **9** were purified by chromatography of the residue on 2 anal. TLC plates (Al_2O_3, $CHCl_3$/MeOH 19:1).

When [1-^{3}H]-**8** was used as substrate (2.0 mg, 0.013 mmol, 1.8 mCi) or as tracer (1.6 μCi, 138 mCi/mmol), the formation of *8-hydroxy-[1-^{3}H]geraniol* ([1-^{3}H]-**9**) was detected by radioactivity scanning. If ^2H- or ^{13}C-labelled substrates were incubated, the products were spotted using a reference standard sprayed with $KMnO_4$ soln. From the distribution of the radioactivity, a reproducible yield of 35% of **9** was calculated corresponding to a specific activity of cytochrome P-450$_{Cath.}$ of 1 nmol converted substrate/min·mg microsomal protein.

[1-(Methoxycarbonyl)(2-^{13}C)ethyl]triphenylphosphonium Iodide (**13**). $^{13}CH_3I$ (7.00 g, 49.0 mmol; 90% ^{13}C enrichment) was added to a soln. of **12** (16.48 g, 49.3 mmol) in 163 ml of abs. AcOEt and heated under reflux for 2 h. On standing at 25° over night, **13** separated as slightly yellow needles which were filtered off and washed twice with 15 ml of warm AcOEt to yield, after drying at 0.01 Torr, 14.31 g (61.3%) of **13** as colourless needles, m.p. 168.9–170.1° (in agreement with [30]). ^1H-NMR (200 MHz): 8.16–7.51 (*m*, 15 H); 6.40 (*m*, 1 H); 3.56 (*s*, 3 H); 1.73 (*ddd*, J = 7.0, J(H,C) = 133, J(H,P) = 18.0, 3 H).

(E)-3,7-Dimethylocta-2,6-dienyl Benzyl Ether (**10**). Geraniol (7.69 g, 49.8 mmol; **8**) was benzylated according to [28] to yield 7.40 g (60.8%) of GLC-pure **10**, b.p. 122°/0.1 Torr. ^1H-NMR (200 MHz): 7.36–7.25 (*m*, 5 H); 5.40

(br. *t*, *J* = 7.0, 1 H); 5.14–5.07 (*m*, 1 H); 4.50 (*s*, 2 H); 4.02 (*d*, *J* = 7.0, 2 H); 2.16–1.99 (*m*, 4 H); 1.68, 1.64, 1.60 (3*s*, 9 H).

(E)-6-Benzyloxy-4-methyl-4-hexenal (**11**). According to [28], 14.91 g (61.0 mmol) of **10** was treated with O_3 to afford, after workup and purification [28], 2.74 g (20.5%) of **11** as a colourless liquid, b.p. 117°/0.01 Torr. ^{1}H-NMR (400 MHz): 9.77 (*t*, *J* = 1.6, 1 H); 7.38–7.27 (*m*, 5 H); 5.40 (br. *t*, *J* = 6.7, 1 H); 4.50 (*s*, 2 H); 4.02 (*d*, *J* = 7.0, 2 H); 2.62–2.33 (*m*, 4 H); 1.66 (*s*, 3 H).

*Methyl (2Z,6E)-8-Benzyloxy-2,6-(2-*methyl-^{13}*C)dimethylocta-2,6-dienoate* ((2-*methyl*-^{13}C)-**14**). Aldehyde **11** (1.05 g, 4.81 mmol) and **13** (1.91 g, 4.00 mmol) were suspended in 6 ml of benzene/oxirane 1:1 and left in a sealed tube for 20 h at 25°. After heating for further 2 h at 75°, the tube was opened, the oxirane removed with a gentle stream of N_2, the residue taken up in Et_2O/pentane 1:1 and filtered through 5 g of SiO_2 to afford 1.25 g of crude (2-*methyl*-^{13}C)-**14**. Chromatography on SiO_2 (pentane/Et_2O 4:1) yielded 811.2 mg (70.1%) of (2-*methyl*-^{13}C)-**14** (95.0% of (2*Z*)- and 3.0% of (2*E*)-isomer and 2% unidentified compounds, according to GLC). ^{1}H-NMR (200 MHz): 7.36–7.25 (*m*, 5 H); 6.77–6.68 (*m*, 1 H); 5.43 (*tq*, *J* = 6.8, 1.3, 1 H); 4.50 (*s*, 2 H); 4.03 (br. *d*, *J* = 6.8, 2 H); 3.71 (*s*, 3 H); 2.37–2.12 (*m*, 4 H); 1.83 (br. *d*, *J*(H,C) = 128.6, 3 H); 1.66 (*d*, *J* = 0.7, 3 H). ^{13}C-NMR (50 MHz): 168.4 (*s*); 141.5 (*s*); 138.9 (*s*); 138.5 (*s*); 128.2 (*s*); 127.8 (*d*, *J*(C,C) = 45.2); 127.7 (*s*); 127.4 (*s*); 71.9 (*s*); 66.4 (*s*); 51.5 (*s*); 38.0 (*s*); 26.8 (*d*, *J*(C,C) = 3.0); 20.5 (*s*, (2*E*)-isomer); 16.3 (*s*); 12.3 (*s*, (2*Z*)-isomer).

*(2Z,6E)-8-Benzyloxy-2,6-(2-*methyl-^{13}*C)dimethylocta-2,6-dienol* ((2-*methyl*-^{13}C)-**15**). Diisobutylaluminium hydride (DIBAH, 20% in toluene; 4.2 mmol) was added dropwise to a soln. of 526.6 mg (1.82 mmol) of (2-*methyl*-^{13}C)-**14** in 15 ml of abs. Et_2O at 0° under Ar. Thereafter, the mixture was stirred for further 15 min, worked up with a sat. *Seignette*-salt soln., and the product isolated by extraction with Et_2O. The combined org. phase was dried over Na_2SO_4, evaporated, and the residue purified by chromatography on SiO_2 (Et_2O/pentane 1:1) to afford 464.4 mg (97.8%) of (2-*methyl*-^{13}C)-**15**, > 98% pure by GLC. ^{1}H-NMR (200 MHz): 7.37–7.26 (*m*, 5 H); 5.44–5.36 (br. *m*, 2 H); 4.50 (*s*, 2 H); 4.02 (*d*, *J* = 6.8, 2 H); 3.98 (*d*, *J*(H,C) = 2.2, 2 H); 2.19–2.05 (*m*, 4 H); 1.66 (*d*, *J*(H,C) = 125.9, 3 H); 1.65 (*s*, 3 H); 1.57 (br. *s*, 1 H, exchanges with D_2O). ^{13}C-NMR (50 MHz): 139.8 (*s*); 138.4 (*s*); 135.0 (*d*, *J*(C,C) = 43.0); 128.2 (*s*); 127.7 (*s*); 127.4 (*s*); 125.3 (*s*); 121.0 (*s*); 72.0 (*s*); 68.6 (*d*, *J*(C,C) = 4.5); 66.4 (*s*); 39.1 (*s*); 25.7 (*d*, *J*(C,C) = 3.3); 16.3 (*s*); 13.6 (*s*); the intensity of the resonance at 21.1 ppm indicates the presence of ≤ 1% of (2*E*)-(2-*methyl*-^{13}C)-**15**.

*(2E,6Z)-3,7-(7-*methyl-^{13}*C)Dimethylocta-2,6-dienyl Benzyl Ether* ((7-*methyl*-^{13}C)-**10**). In analogy to [31], 180 mg (1.78 mmol) of Et_3N were added at −70° under Ar to a soln. of 115.0 mg (0.44 mmol) of (2-*methyl*-^{13}C)-**15** in 1.5 ml of abs. Et_2O followed by septum injection of 56.3 mg (0.5 mmol) of CH_3SO_2Cl in 0.4 ml of abs. Et_2O within 10 min. After stirring for 1 h at −70° and 1 further h at −30°, the mixture was slowly warmed up to −10° and stirred for 1 additional h. Subsequent operations were carried out at 0–4°: dilution with Et_2O and washing of the org. layer with 5% HCl, sat. $NaHCO_3$, and sat. NaCl soln., afforded, after drying over Na_2SO_4, evaporation, and drying at 0.01 Torr for 1 h, 146 mg of crude (2-*methyl*-^{13}C)-**16**. The slightly yellow oil was immediately dissolved in 0.3 ml of abs. THF under Ar and added to a suspension of 60 mg (1.56 mmol) of $LiAlH_4$ in 1.7 ml of abs. THF at 25°. After stirring for 2 h, the mixture was worked up by addition of a sat. *Seignette*-salt soln., followed by Et_2O extraction and drying of the combined org. layers with Na_2SO_4. Subsequent evaporation and chromatography of the crude material on SiO_2 (pentane/Et_2O 9:1) yielded 80.6 mg (75.0%) of pure (7-*methyl*-^{13}C)-**10** as a colourless oil. ^{1}H-NMR (200 MHz): 7.37–7.25 (*m*, 5 H); 5.41 (*tq*, *J* = 6.8, 1.3, 1 H); 5.11 (*m*, 1 H); 4.51 (*s*, 2 H); 4.04 (br. *d*, *J* = 6.8, 2 H); 2.19–2.00 (*m*, 4 H); 1.68 (br. *s*, 3 H); 1.66 (br. *s*, 3 H); 1.62 (br. *d*, *J*(H,C) = 125, 3 H).

*(7-*methyl-^{13}*C)Geraniol* ((7-*methyl*-^{13}C)-**8**). To a soln. of 80.6 mg (0.33 mmol) of (7-*methyl*-^{13}C)-**10** in 8 ml of abs. THF/abs. $EtNH_2$ 1:7, *ca.* 10 mg of freshly cut Li wire (1% Na) were added in small pieces under Ar at −70°. After 80 min stirring, a faint blue colour appeared accounting for a slight excess of metal in soln. The reaction was immediately quenched by addition of 0.5 ml of MeOH and poured into a sat. NH_4Cl soln., which was extracted 4 times with CH_2Cl_2. The combined org. layers were dried over Na_2SO_4, evaporated, and the oily residue purified by chromatography on SiO_2 (Et_2O/pentane 1:1) to yield 45.0 mg (87.8%) of (7-*methyl*-^{13}C)-**8** as a colourless liquid, 95.5% pure by GLC. ^{1}H-NMR (400 MHz): 5.42 (*tsext.*, *J* = 7.0, 1.3, 1 H); 5.11 (*m*, 1 H); 4.16 (br. *d*, *J* = 7.0, 2 H); 2.14–2.02 (*m*, 4 H); 1.68 (*s* and br. *d*, *J*(H,C) = 1.5, 6 H); 1.61 (br. *d*, *J*(H, C) = 125, 3 H); 1.25 (br. *s*, 1 H, exchanges with D_2O). ^{13}C-NMR (50 MHz): 139.3 (*s*); 131.5 (*d*, *J*(C,C) = 42.6); 123.8 (*d*, *J*(C,C) = 1.6); 123.4 (*s*); 59.1 (*s*); 39.5 (*s*); 26.3 (*d*, *J*(C,C) = 3.5); 25.5 (*d*, *J*(C,C) = 4.3, (6*E*)-isomer < 2%); 17.5 (*s*, (6*Z*)-isomer > 98%); 16.1 (*s*). EI-MS: 124 (6, $M^{+\cdot}$ − 31), 94 (8), 93 (33), 92 (9), 91 (8), 84 (8), 81 (5), 80 (9), 79 (8), 77 (7), 71 (8), 70 (100), 69 (22), 68 (25).

*(2Z,6E)-8-Benzyloxy-2,6-(2-*methyl-^{13}*C)dimethyl(1,1-*^{2}*H$_2$)octa-2,6-dienol* ((2-*methyl*-^{13}C,1,1-^{2}H$_2$)-**15**). A soln. of 144.0 mg (0.50 mmol) of (2-*methyl*-^{13}C)-**14** in 0.5 ml of abs. Et_2O was slowly added to a suspension of 84.0 mg (2.0 mmol) of $LiAlD_4$ in 2 ml of abs. Et_2O at 0° under Ar. After 2 h stirring at 25°, the product was isolated and

purified as described above affording 92.25 mg (70.0%) of GLC-pure (2-*methyl*-^{13}C, 1,1-$^{2}H_2$)-**15** as a colourless liquid. ^{1}H-NMR (200 MHz): identical resonances as (2-*methyl*-^{13}C)-**15**, but lacking the *d* at 3.98 ppm (2 H–C(1)).

(7-methyl-^{13}*C,8,8,8-*$^{2}H_3$*)Geraniol* ((7-*methyl*-^{13}C,8,8,8-$^{2}H_3$)-**8**). In the presence of $EtNH_2$, (2-*methyl*-^{13}C,1,1-$^{2}H_2$)-**15** (92.0 mg, 0.35 mmol) was treated with 45.8 mg (0.40 mmol) of CH_3SO_2Cl and the resulting (2-*methyl*-^{13}C,1,1-$^{2}H_2$)-**16** subsequently reduced with 60.0 mg (1.43 mmol) of $LiAlD_4$ in abs. THF as described above to yield, after usual workup and purification, 56.2 mg (64.6%) of (7-*methyl*-^{13}C,8,8,8-$^{2}H_3$)-**10**. ^{1}H-NMR (200 MHz): the resonance at 1.68 (3 H–C(8)) of (7-*methyl*-^{13}C)-**10** is lacking.

Reductive ether cleavage was performed as described above to afford, after usual workup and chromatography, 24.0 mg (66.4%) of (7-*methyl*-^{13}C,8,8,8-$^{2}H_3$)-**8**, 96.0% pure by GLC. ^{1}H-NMR (400 MHz): in contrast to (7-*methyl*-^{13}C)-**8**, only 2 CH_3 resonances; 1.68 (br. *s*, CH_3–C(3)); 1.60 (br. *d*, *J*(H,C) = 125, $^{13}CH_3$–C(7)). EI-MS: 158 (1, $M^{+\cdot}$), 140 (3, $M^{+\cdot} - 18$), 127 (8), 111 (7), 93 (16), 84 (9), 80 (7), 73 (100), 72 (18), 71 (8), 70 (5), 68 (16).

*(7-*methyl-^{13}*C,8,8-*$^{2}H_2$*)Geraniol* ((7-*methyl*-^{13}C,8,8-$^{2}H_2$)-**8**). The (2-*methyl*-^{13}C,1,1-$^{2}H_2$)-**16** (100.5 mg, 0.294 mmol; see above) was reduced with 51.0 mg (1.34 mmol) of $LiAlH_4$ in 2 ml of abs. THF yielding 60.1 mg (82.6%) of (7-*methyl*-^{13}C,8,8-$^{2}H_2$)-**10**. ^{1}H-NMR (200 MHz): characteristic br. *s* at 1.65 (4 H, H–C(8), CH_3–C(3)), in contrast to (7-*methyl*-^{13}C)-**10**.

Reductive cleavage by Li/1% Na afforded, after usual workup and chromatography, 25.8 mg (67.6%) of (7-*methyl*-^{13}C,8,8-$^{2}H_2$)-**8**, 94.7% pure by GLC. ^{1}H-NMR (400 MHz): in contrast to the undeuterated and trideuterated geraniols described above, br. *m* at 1.66 (1 H, H–C(8)). EI-MS: 157 (1, $M^{+\cdot}$), 139 (3, $M^{+\cdot} - 18$), 126 (8), 111 (6), 93 (18), 84 (9), 80 (7), 73 (7), 72 (100), 71 (22), 70 (7), 69 (7), 68 (18), 67 (9).

[1-^{3}H*]Geraniol* ([1-^{3}H]-**8**). A GLC-pure (97.1%) sample of citral (**17**) was prepared in 89% yield from geraniol (**8**) by MnO_2 oxidation in hexane according to [38]. A soln. of 29.82 mg (0.196 mmol) of **10** in 10 ml of abs. i-PrOH was treated at –10° under Ar with 1.6 mg $NaBH_3[^3H]$ (0.042 mmol, 25 mCi; according to *Amersham*, UK). After 0.5 h at 0°, the solvent was evaporated and the residue purified by chromatography on SiO_2 (pentane/Et_2O 6:4) to yield 20.2 mg of [1-^{3}H]-**8** (78% from $NaBH_3[^3H]$, 18.15 mCi, 138 mCi/mmol), > 98% pure by GLC and radio-TLC scanning.

1,2-Dithian-4,5-diol (**23**). O_2 was bubbled for 15 min at 25° through a soln. of 100.0 mg (0.658 mmol) of dithiothreitol (DTT; = 1,4-dimercaptobutan-2,3-diol; **22**) in 2.8 ml of EtOH/33% aq. NH_3 8:1. After stirring for 5 h, evaporation of the solvent, and crystallisation of the solid residue from $CHCl_3$, 76.0 mg (76.0%) of **23** were isolated as colourless needles, m.p. 122.0–125.0°, in agreement with [39]. ^{1}H-NMR (400 MHz, DMSO): 5.21 (*d*, *J* = 3.9, 2 H, exchanges with D_2O); 3.30 (*dd*, *J* = 13, 7, 2 H); 3.02 (*d*, *J* = 13, 2 H); 2.70 (*dd*, *J* = 13, 7, 2 H). EI-MS: 154 (4, $M^{+\cdot} + 2$), 152 (38, $M^{+\cdot}$), 134 (8, $M^{+\cdot} - 18$), 108 (53), 87 (17), 80 (10), 79 (29), 78 (6), 77 (8), 74 (6), 73 (5), 70 (20), 69 (6), 66 (5), 64 (45, S_2), 60 (20).

8-Hydroxygeraniol (= *(*E,E*)-2,6-Dimethylocta-2,6-dien-1,8-diol;* **9**). Allylic oxidation of 901.6 mg (4.60 mmol) of geranyl acetate (**18**) was performed according to [32] by adding **18** at 25° to a dark red soln. of 1.22 g (5.0 mmol) of *N,N*,1,1-Tetramethyl-2-[(selenino)oxy]ethylamine oxide (**19**) and 1.135 g (5.0 mmol) of DDQ in 25 ml of abs. $CHCl_3$/DMSO 9:1. The reaction was complete after 30 min stirring at 25°. The soln. was filtered through SiO_2 (pentane) and a 1:1 mixture **20**/**21** eluted with 50 ml of Et_2O/pentane 1:1 affording 704.0 mg (90%) after complete removal of the solvents at 0.01 Torr. The mixture was dissolved in abs. Et_2O under Ar, and 10 ml of DIBAH (20% in toluene) were added at 0° within 5 min. After stirring at 25° for 1 h, usual workup, and chromatography on SiO_2 (Et_2O), 541.9 mg (85%) of **9** were obtained, 96.7% pure by GLC. ^{1}H-NMR (400 MHz): 5.42–5.35 (*m*, 2 H); 4.15 (*d*, *J* = 6.9, 2 H); 3.99 (*s*, 2 H); 2.20–2.05 (*m*, 4 H); 1.68 (*s*, 3 H); 1.66 (*s*, 3 H). ^{13}C-NMR (50 MHz): 138.4 (*s*); 135.0 (*s*); 125.0 (*s*); 123.8 (*s*); 68.4 (*s*); 59.0 (*s*); 38.9 (*s*); 25.5 (*s*); 16.0 (*s*); 13.6 (*s*). EI-MS: 155 (< 1, $M^{+\cdot} - 15$), 152 (< 1, $M^{+\cdot} - 18$), 134 (4, $M^{+\cdot} - 36$), 121 (12), 109 (10), 94 (15), 84 (35), 81 (20), 68 (70), 43 (100). CI-MS: 172 (4, $M^{+} + 2$), 153 (12), 135 (100), 109 (13), 107 (15), 95 (32), 93 (18), 81 (29), 69 (14).

Identification of 8-Hydroxy[1-^{3}H*]geraniol* ([1-^{3}H]-**9**) *from the Incubation of [1-*^{3}H*]-***8** *with Cytochrome P-450*$_{Cath.}$. A soln. of 2.0 mg (0.013 mmol, 1.8 mCi) of [1-^{3}H]-**8** in 1 ml of acetone was incubated under standard conditions with 120 mg of microsomal protein *a)* in the presence of DTT (**22**) or *b)* in the absence of DTT (**22**). Aliquotes (0.01 ml, 1%) were taken from the unpurified $CHCl_3$ extract of these assays and silylated for 3 min in 0.02 ml of pyridin in the presence of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Coupled GLC/gas proportional counter: peak matching of the FID signal and the ^{3}H counter was observed in both assays *a)* and *b)* at 11.0 min for [1-^{3}H]-**25** and at 17.8 min for [1-^{3}H]-**24**; retention times identical to those of the non-radioactive compounds. GLC/MS for *a)*: the bissilyl ether of the cyclic disulfide **23** (main component) covered the peak of [1-^{3}H]-**24**. GLC/MS for *b)*: MS of the first eluting GLC peak identical to MS of **25**, MS of the second peak identical to the MS of **24**. *[1-*^{3}H*]Geranyl Trimethylsilyl Ether* ([1-^{3}H]-**25**): EI-MS: 226 (3, $M^{+\cdot}$), 211 (9), 183 (9), 169 (16), 156 (22), 143 (47), 121 (63), 93 (53), 69 (100).

[1-^{3}H]Geran-1,8-diyl Bis(trimethylsilyl) Diethylether (= *(*E,E*)-2,6-Dimethyl[8-^{3}H]octa-2,6-dien-1,8-diyl Bis (trimethylsilyl) Diether*; [1-^{3}H]-**24**): EI-MS: 314 (< 1, $M^{+\cdot}$), 299 (< 1), 231 (7), 224 (7), 209 (18), 182 (14), 169 (20), 156 (45), 147 (64), 141 (41), 75 (100).

*Incubation of (7-*methyl-^{13}*C)*-**8** *with Cytochrome P-450$_{Cath.}$*. A soln. of 1.0 mg of (7-*methyl*-^{13}C)-**8** in 0.5 ml of acetone was incubated under standard conditions with 60 mg of microsomal protein for 30 min. After extraction and chromatography, **23**, (7-*methyl*-^{13}C)-**9**, and (7-*methyl*-^{13}C)-**8** were separated. Further purification of the latter on SiO_2 (Et_2O/pentane 1:1) afforded spectroscopically unchanged (7-*methyl*-^{13}C)-**8**. ^{1}H-NMR: identical with that of the starting material. ^{13}C-NMR: only 1 enriched signal at 17.6 ppm (intensity 200), no signal at 25.5 ppm.

GLC-pure (7-*methyl*-^{13}C)-**9**: ^{1}H-NMR (400 MHz): 4.18 (*d*, *J* = 6.9, 2 H); 4.01 (*d*, *J* = 2, 2 H); 1.69 (*s*, 3 H); 1.67 (br. *d*, *J*(H,C) = 126.3, 3 H). ^{13}C-NMR (50 MHz): enhanced signal at 13.7 ppm (intensity 137), no signal at 68.4 ppm.

*Intermolecular Isotope Effect: Incubation of (7-*methyl-^{13}*C)*-**8** *and (7-*methyl-^{13}*C,8,8,8-*$^2H_3$*)*-**8** *with Cytochrome P-450$_{Cath.}$*. For each of the following exper. 0.5 ml of a standard soln. was used, consisting of 5.70 mg of (7-*methyl*-^{13}C)-**8**, 5.80 mg of (7-*methyl*-^{13}C,8,8,8-$^2H_3$)-**8**, and 0.02 mg of [1-^{3}H]-**8** in a final volume of 5.5 ml of acetone. Incubations were performed under standard conditions with P-450$_{Cath.}$, but quenched after 5, 10, 15, and 30 min. After usual workup and chromatography, the isolated mixtures (7-*methyl*-^{13}C)-**9**/(7-*methyl*-^{13}C,8,8-$^2H_2$)-**9** were analysed. ^{1}H-NMR (400 MHz): all identical except for the ratio of peak areas of the resonances at 4.18 (H–C(1)) and 4.01 ppm (H–C(8)); the integration of the latter signal reflects the concentration of (7-*methyl*-^{13}C)-**9**; peak area at 4.18/peak area at 4.01 (average of several exper.) = 2.89 (5 min), 2.33 (10 min), 2.15 (15 min), 1.98 (30 min); k_H/k_D = 0.50 (5 min), 0.75 (10 min), 0.87 (15 min), 1.02 (30 min); all values ±0.04.

*Incubation of a Mixture (7-*methyl-^{13}*C)*-**9**/*(7-*methyl-^{13}*C,8,8-*$^2H_2$*)*-**9**/*[1-^{3}*H]*-**9** *with Cytochrome P-450$_{Cath.}$*. 8-Hydroxygeraniol (**9**; 0.6 mg), which according to ^{1}H-NMR consisted of a 1:1 mixture (7-*methyl*-^{13}C)-**9**/(7-*methyl*-^{13}C,8,8-$^2H_2$)-**9**, containing 8.68 × 10^5 dpm [1-^{3}H]-**9** was incubated under standard conditions for 30 min with P-450$_{Cath.}$. After usual workup, 1.19 × 10^4 dpm ^{3}H activity (1.4%) was measured in the lyophylised H_2O and 8.29 × 10^5 dpm ^{3}H activity (98.6%) was determined in the org. layer; total recovery 8.41 × 10^5 dpm ^{3}H (96.9%). Radio TLC-scanning (Al_2O_3, $CHCl_3$/CH_3OH 19:1, silica gel, Et_2O) revealed that no radioactive compounds other than [1-^{3}H]-**9** were present. In these TLC systems, all possible ω-oxidation products of **9** like the hydroxyaldehydes, the dialdehyde, the mono- and diacids displayed R_f values clearly different from [1-^{3}H]-**9**, as shown by comparison with synthetic reference samples; this is also confirmed by GLC. After usual chromatography, a sample of 8-hydroxygeraniol was obtained, which according to ^{1}H-NMR spectroscopy (400 MHz) consisted of a 1:1 mixture (7-*methyl*-^{13}C)-**9**/(7-*methyl*-^{13}C,8,8-$^2H_2$)-**9** (peak area at 4.18/peak area at 4.01 = 1.98).

*Intramolecular Isotope Effect: Incubation of (7-*methyl-^{13}*C,8,8-*$^2H_2$*)*-**8** *with Cytochrome P-450$_{Cath.}$*. A soln. of 1.0 mg of (7-*methyl*-^{13}C,8,8-$^2H_2$)-**8** in 0.5 ml of acetone was incubated under standard conditions and the mixture (7-*methyl*-^{13}C,8,8-$^2H_2$)-**9**/(7-*methyl*-^{13}C,8-^{2}H)-**9** isolated by usual workup and chromatography. ^{1}H-NMR (400 MHz): peak area at 4.18 (H–C(1))/peak area at 4.01 (H–C(8)) = 10 accounting for a molar ration (7-*methyl*-^{13}C,8,8-$^2H_2$)-**9**/(7-*methyl*-^{13}C,8-^{2}H)-**9** = 4.0. Since the removal of ^{2}H is statistically favoured by a factor of 2, k_H/k_D = 8.0 ± 0.5 (average from 4 incubations).

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