156. Fermentation of Fragrances: Biotransformation of β-Ionone by *Lasiodiplodia theobromae*

by **Victor Krasnobajew** and **Daniel Helmlinger**

Givaudan Research Company Ltd., CH-8600 Dübendorf-Zürich

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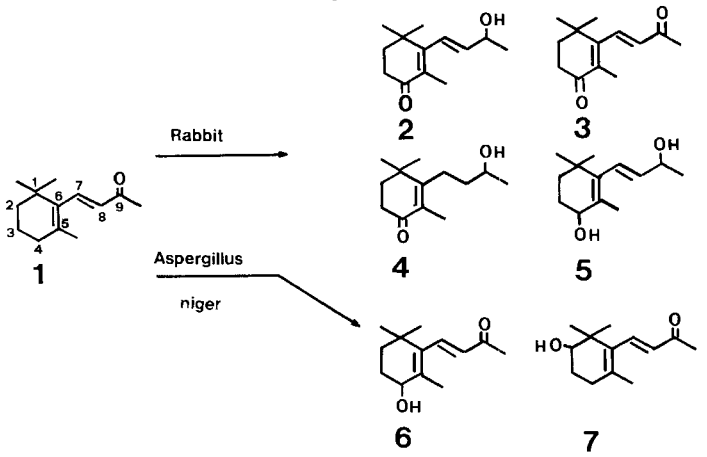
Summary

Pre-grown mycelia of *Lasiodiplodia theobromae* ATCC 28570 transform β-ionone (**1**) into a large variety of metabolites, by mainly degrading the side-chain of the β-ionone molecule by a C_2-unity. The enzyme system responsible for this degradation is proposed to be an oxygenase, which gives rise to the formation of the main product β-cyclo-homogeraniol (**8**) in analogy to a *Baeyer-Villiger* oxidation. Further enzymic actions of *Lasiodiplodia* such as hydrogenations and hydroxylations lead to an accumulation of several not yet described β-ionone metabolites.

Introduction. – Ionones and their derivatives are widely distributed in nature, they are important constituents of many essential oils [1], and they are thought to originate in nature from carotenes by complex enzymatic actions [2] or degradations [3]. Relatively little is known from literature about the metabolic fate of ionones in biological systems. The metabolism of β-ionone (**1**) in rabbits was studied by several investigators [4–6], and four oxigenated β-ionone metabolites

Scheme 1



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were identified (**2**–**5**). *Mikami et al.* [7] described 1978 the hydroxylation of β-ionone by *Aspergillus niger*. The major transformation products were identified as (*R*)-4-hydroxy-β-ionone (**6**), and (*S*)-2-hydroxy-β-ionone (**7**). No degradation of the β-ionone molecule was observed.

In the course of our studies on fermentations of fragrant compounds we have found that *Lasiodiplodia theobromae* ATCC 28570 transformed β-ionone in a considerably different way, leading to an essential oil-type product.

The present publication describes the formation of the β-ionone metabolites, their isolation and identification as well as the chemical synthesis of various metabolites.

Results and discussion. – 1. *Fermentation.* In the course of our screen for appropriate microorganisms capable to transform β-ionone, we found that the closely

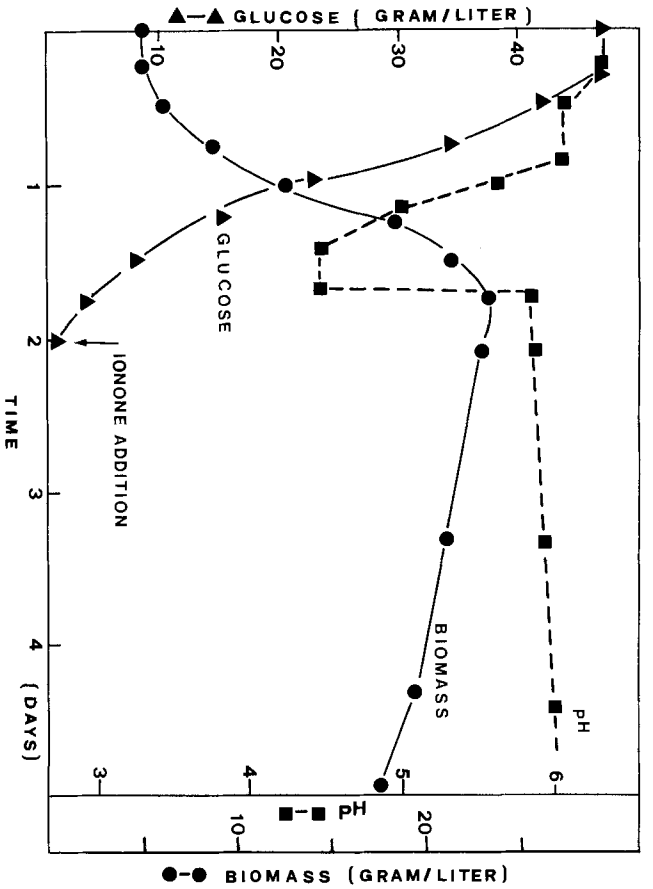


Fig. 1. *Growth of* Lasiodiplodia theobromae *and fermentation of β-ionone. Lasiodiplodia theobromae* ATCC 28570 was grown in a 75 l fermenter equipped with axial flow-impeller with draft-tube. The 45 l growth medium consisted of: 5% glucose, 1% cornsteepliquor, 0.05% KCl, 0.1% KH_2PO_4 and 0.1% $NaNO_3$. The aeration was 20 l/min and the growth temperature 28°. β-Ionone addition was started after glucose was practically consumed.

related genera of *Botryodiplodia*, *Botryoshaeria* and *Lasiodiplodia* were fairly resistant to β-ionone in respect to cell-lysis, and developed a considerably higher transformation capacity compared to other tested microorganisms. Especially *Lasiodiplodia theobromae* ATCC 28570, recently also listed as *Diplodia gossipina* in the *American Type Culture Collection Catalogue* was found most suitable for our biotransformation studies of β-ionone.

The fermentation of β-ionone has to be done with pre-grown cultures because β-ionone inhibits growth and leads to lysis of the cells. Usually mycelia cultures were employed which were at the end of their logarithmic growth phase. This point was reached after 20 to 50 h, depending on the growth medium in shake cultures or in fermenters. In *Figure 1* a characteristic growth curve of the fungus in a 75 l fermenter is shown. The growth of the fungus was followed by measuring the glucose consumption from the medium, and by determining the formation of biomass. As shown in *Figure 1*, after about 48 h glucose is practically consumed, and the biomass has reached more than 20 g dried matter per liter. At this point the addition of β-ionone is started. To ensure that the organisms are not overloaded with the toxic substrate the addition is done portionwise or continuously in small amounts. The formation of metabolites is persued by gas chromatography (GC.) and thin-layer chromatography (TLC.).

After 3 to 4 days the transformation capacity of the mycelia decreases considerably, and the fermentation broth is extracted. Under optimal conditions mycelia of *Lasiodiplodia theobromae* ATCC 28570 transform about 10 g β-ionone per liter culture. The extracted metabolites represent a brownish oil with a pleasant sweetish odor which reminds of tobacco flavor.

2. *Analysis of the β-ionone transformation product.* In *Figure 2* a characteristic gas chromatogram is shown of the extracted crude oil from a 45 l fermentation run of β-ionone. As can be seen the oil represents a complex mixture of numerous metabolites of which three compounds, **8**, **10** and **12**, dominate in the product.

In order to elucidate the structure of the compounds we have isolated the single compounds by chromatography of the crude oil on silicagel. Usually we isolated first some starting material (**1**) and some 7,8-dihydro-β-ionone (**1a**). But these compounds can be absent when the biotransformation is conducted over longer periods of time without further addition of β-ionone. We eluted with hexane/ether 1:1 an alcohol with molecular weight of 168. The ^{1}H-NMR. spectrum of this compound showed the presence of a geminal dimethyl at 1.06 ppm, a methyl on double bond at 1.7 ppm, and two triplets at 2.4 and 3.66 ppm corresponding to an A_2B_2-pattern.

The structure **8** for this compound ccould be confirmed easily by reduction of the known β-cyclo-homocitral (**27**) [13] with lithium aluminium hydride. The resulting β-cyclo-homogeraniol (**8**) is identical with the product isolated from the microbial transformation product.

The following product eluted in the chromatography was the diene **13** which showed in the UV. a λ_{max} at 262 mμ. The IR., ^{1}H-NMR. and MS. of this compound confirmed the structure **13**. By eluting with hexane/ether 8:2 we could isolate an enone with a molecular weight of 182; UV.: λ_{max} at 246 mμ.

According to IR., ^{1}H-NMR. and ^{13}C-NMR. this compound has the structure **12**. With hexane/ether 1:9 we eluted a mixture of two compounds **16** and **17** which could be separated later by a second chromatography on silicagel.

By further eluting with pure ether we obtained one main compound 4-hydroxy-β-cyclo-homogeraniol (**10**) and a minor compound (**19**) purified by a second chromatography. Treating 4-hydroxy-β-cyclo-homogeraniol (**10**) with $KHSO_4$ in DMSO at 80° we obtained a mixture of 3,4-didehydro-β-cyclo-homogeraniol (**13**) and the ether **15**. The presence of ether **15** in the crude transformation product could be verified by GC.-MS. investigation.

The minor component eluted with ether was shown to be 2-hydroxy-4-oxo-7,8-dihydro-β-ionone (**19**) by its spectral data. The ^{1}H-NMR. (400 MHz) showed

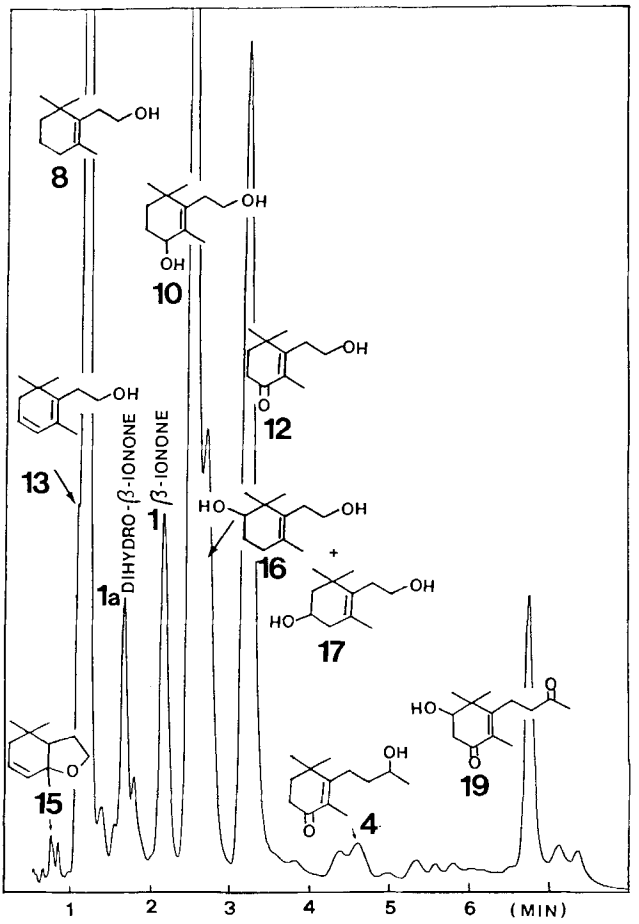


Fig. 2. *Gas chromatogram of β-ionone metabolite mixture produced by* Lasiodiplodia theobromae. Conditions: Packed glass column (3 mm × 2 m) with OV-1, 5% on *Gaschrom Q* (60–80 mesh), 150° isotherm 5 min, then temperature increase of 30°/min.

the presence of an equatorial hydroxy-group at C(2) (characterized by a quartet at 3.84 ppm, $J(H_{eq}-C(3), H_{ax}-C(2))=4.5$, $J(H_{ax}-C(2), H_{ax}-C(3))=9$). The presence of a hydroxy-group in α-position to the geminal dimethyl group was confirmed by ^{13}C-NMR. Here we could determine an α-effect of 5.8 ppm on C(2) and a γ-effect of -1.9 and -6 ppm on the gem. dimethyl group. The ^{13}C-NMR. of 4-oxo-7,8-dihydro-β-ionone was available from previous microbial transformation of β-ionone by *Rhizopus arrhizus*.

By treatment of 2-hydroxy-4-oxo-7,8-dihydro-β-ionone (**19**) with $KHSO_4$ in DMSO we obtained the 4-oxo-2,3-didehydro-7,8-dihydro-β-ionone (**21**).

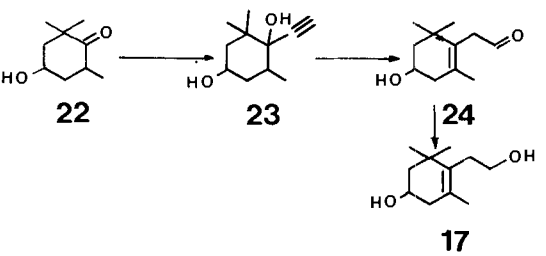
The above mentioned mixture of two compounds **16** and **17** eluted with hexane/ether 9:1 was rechromatographed on silicagel eluting with ethyl acetate/CH_2Cl_2 1:9. The compound eluting first was shown to be 3-hydroxy-β-cyclo-homogeraniol (**17**) by its spectral data (IR., ^{1}H-NMR., ^{13}C-NMR. and MS.). Latter structure could be confirmed by a synthesis of **17** starting with 4-hydroxy-2,2,6-trimethyl-cyclohexanone (**22**, *Scheme 2*). Treatment of **22** with sodium acetylide in DMF furnished the diol **23** which subsequently was rearranged to 3-hydroxy-β-cyclo-homocitral (**24**) by the action of tris(triphenylsilyl)-vanadate in toluene. Reduction of 3-hydroxy-β-cyclo-homocitral with lithium aluminum hydride yielded 3-hydroxy-β-cyclo-homogeraniol (**17**) the spectral data of which were identical with those of **17** isolated from the metabolite mixture.

The second compound eluted with ethyl acetate/CH_2Cl_2 1:9 was identified to be 2-hydroxy-β-cyclo-homogeraniol (**16**). The location of a hydroxy-group at the C(2)-position was confirmed by a quartet at 3.48 ppm, $J=9$ and 3.3 Hz in a 400-MHz-^{1}H-NMR. The latter data are in accordance with the NMR. data of the 2-hydroxy-β-ionone metabolite (**19**).

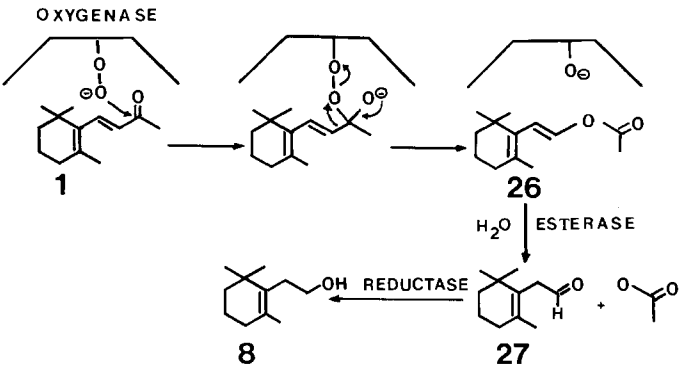
3. *Formation of the β-ionone metabolites.* As was demonstrated in *Scheme 1* *Aspergillus niger* introduces a hydroxy function into β-ionone without degrading the molecule, where as *Lasiodiplodia* mainly degrades β-ionone by shortening the side chain by a C_2-unity *(Fig. 2)*. This process leads to the main compound β-homo-cyclogeraniol (**8**). On the other hand a hydrogenation results in 7,8-dihydro-β-ionone **1a**, and a concomittant hydroxylation of the alcohol **8** yields the diols **10**, **16** and **17**. The hydroxylation of dihydro-β-ionone (**1a**) probably results in compounds **4** and **19**.

These enzymatic actions are performed by intracellular or membrane-bound enzymes of *Lasiodiplodia*. The culture supernatant does not show enzymic activity.

Scheme 2



Scheme 3



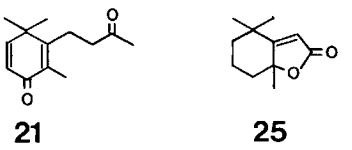
An oxygenase-type enzyme system is proposed to be responsible for the degradation of the ionone side chain. In fact such conversions are well documented in microbial steroid transformation. Several research groups [8] [9] describe already 1953 the side chain degradation of C_{21}-steroids by fungi, leading ultimately to lactones or esters, where the etheral O-atom drives from air oxygen.

The enzymatic oxygen insertions are widely distributed in nature, and are similar to the chemical *Baeyer-Villiger* (**10**) oxidation.

In *Scheme 3* a mechanism for the β-ionone degradation is proposed in analogy to the *Baeyer-Villiger* oxidation and to enzymic investigations of *Prairie & Talalay* [11] who used a soluble enzyme system from *Penicillium lilacinum* in combination with NADPH and molecular oxygen for steroid degradation. Such oxygenase-type enzyme attacks the keto-group, in our case that of β-ionone, by forming a peroxide intermediate, which rearranges according to *Baeyer-Villiger* to the enol ester **26**. Subsequently the enol ester **26** is hydrolyzed by an esterase to form β-cyclo-homocitral **27** and acetic acid, the aldehyde **27** is instantly reduced by a reductase to the corresponding alcohol **8**. The latter two reaction steps we could confirm by incubating first the synthetic ester **26** with *Lasiodiplodia*. The ester-cleavage proceeds rapidly and yields β-cyclo-homogeraniol **8** as the main product. The minor products are the metabolites 2-hydroxy-, 3-hydroxy- and 4-hydroxy-β-cyclo-homogeraniol (**16**, **17** and **10**), furthermore 4-oxo-β-cyclo-homogeraniol (**12**), and a compound not isolated from the metabolite mixture of β-ionone: dihydro-actinidiolide (**25**, *Scheme 4*).

The presence of the latter compound **25** in the transformation product mixture was however verified by GC.-MS. investigation. On the other hand the aldehyde **27** is reduced by *Lasiodiplodia* to the alcohol **8**. This is probably the reason why the

Scheme 4



aldehyde **27** is not found in the metabolite mixture. By incubation the synthetic aldehyde **27** with a pre-grown culture of *Lasiodiplodia* we could identify the same metabolites **17**, **16** and **10**, besides the main transformation product β-cyclo-homogeraniol **8**. Dihydroactinidiolide **25** was also characterized in the mixture.

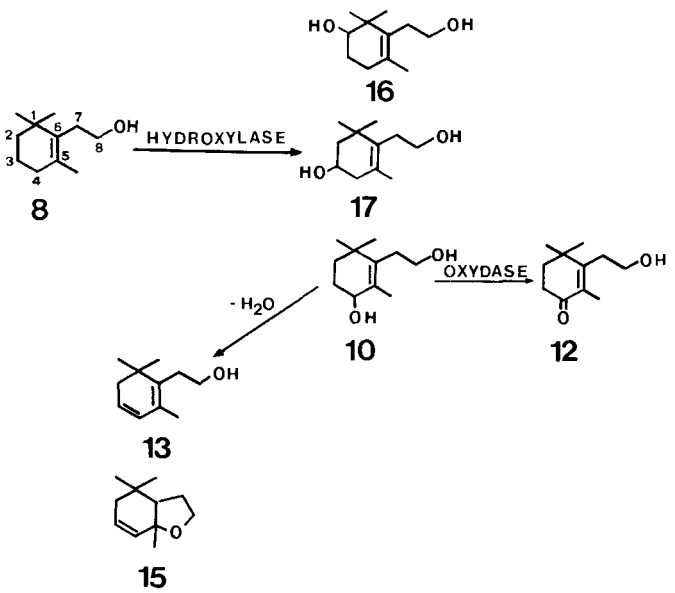
Enzymic actions on β-cyclo-homogeraniol are demonstrated in *Scheme 5*. After enzymic degradation of β-ionone to the alcohol **8** a hydroxylase enzyme system introduces a hydroxy function preferably into the activated allylic position of **8** leading to the main diol **10**. On the other hand the enzymic hydroxylation does not seem specifically restricted to the allylic position as also the remaining two positions of the ring structure are attacked leading to the diols **16** and **17**.

Looking at the variety of β-ionone metabolites at the end of the fermentation process, we were wondering whether the composition of metabolites remains uniform during the whole fermentation period. In *Figure 3* a gas chromatogram of an extracted probe at the early stage of β-ionone fermentation is shown. This is *ca.* 6 h after the first β-ionone addition to the fungus growing at the end of logarithmic growth.

As can be seen this pattern of metabolites differs considerably from that one at the end of fermentation as demonstrated in *Figure 2*. Major compounds are oxigenated β-ionone metabolites **4** and **6** which result from hydroxylation. Metabolite **4** was already reported to be produced by rabbits. Both products disappear practically in the later phase, and could yield by further hydroxylation finally 2-hydroxy-4-oxo-7,8-dihydro-β-ionone (**19**) which is found in considerable quantities in the end product.

The degradation product β-cyclo-homogeraniol (**8**) and its allylic oxidation products **10** and **12** are already present but in much smaller amounts. This finding

Scheme 5



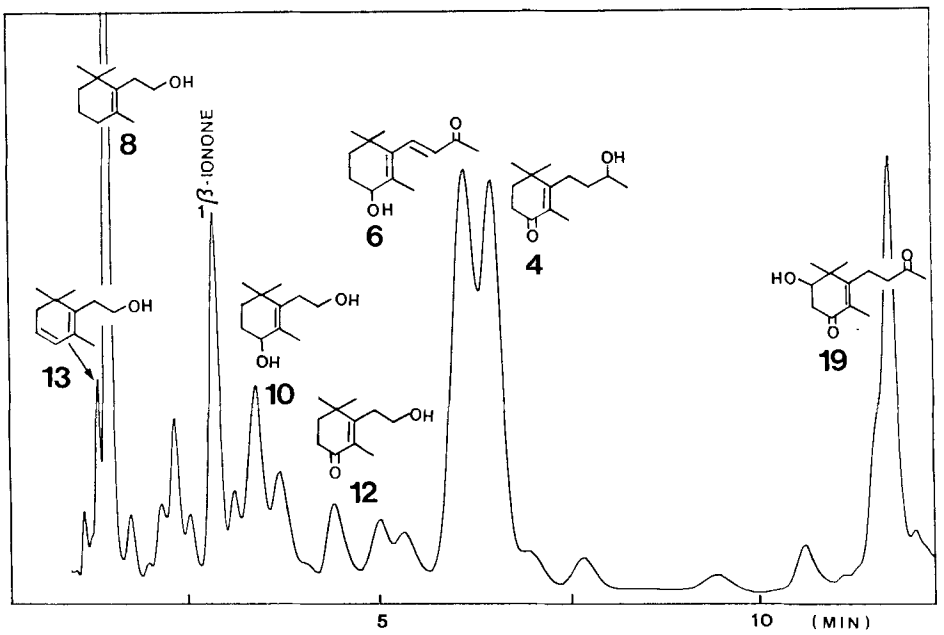


Fig. 3. *Gas chromatogram of β-ionone metabolites produced by* Lasiodiplodia theobromae *at early stage of fermentation* (6 h after first β-ionone addition). Conditions: Packed glass column (3 mm × 2 m) with OV-1, 5% on *Gaschrom Q* (60–80 mesh), 145° isotherm 10 min, then temperature increase of 30°/min.

indicates that at the beginning of β-ionone fermentation hydroxylases are predominantly active. The oxygenase-type enzymes which are responsible for the *Baeyer-Villiger*-type oxidation have to be induced. This finding is more pronounced in shake-cultures where the oxygen transfer is not always optimal. In the latter case the appearance of the degradation product β-cyclo-homogeraniol (**8**) can take 10 to 20 h.

Conclusion. – The fermentation of β-ionone by suitable microorganisms such as fungi from the genus *Lasiodiplodia* yields complex mixtures of metabolites which are chemically not yet accessible. Furthermore it is shown that an essential oil-type product can be obtained by fermentation of a readily available synthetic fragrance. The described enzymic actions on the β-ionone molecule could help to explain the formation of metabolites from carotenes in nature.

The authors are indebted to the colleagues from the analytical division Dr. *E. Billeter, J. Märki, G. Zarske* and Dr. *N. Neuner* for their expert assistance, and to *R. Kaiser* for fruitful discussions.

Experimental Part

(with the collaboration of *S. Rytkönen*)

General remarks. Preparative column-chromatography was performed on Silicagel *Merck 60* (particle size 0.063–0.200 mm). Gas chromatography (GC.) was run on a *Carlo Erba* Fractovap GI. with FID. Recording of spectra was carried out on the following apparatus. UV.: *Beckman* DB-G

spectrophotometer (λ_{max} in nm, ε in parentheses). – IR.: *Perkin-Elmer* 257 spectrophotometer; characteristic band positions are given in cm^{-1}. – ^{1}H-NMR.: *Varian* EM-360, *Varian* XL-100, *Bruker* WH-360 and WH-400. Measurements were run in $CDCl_3$ with TMS (0 ppm) as internal standard. Abbreviations: *s* = singlet; *d* = doublet; *t* = triplet; *qa* = quartet; *m* = multiplet; br. = broad; *J* = apparent coupling constant in Hz. – ^{13}C-NMR.: *Varian* XL-100A at 25.2 MHz (measurements in $CDCl_3$ with TMS (0 ppm) as internal standard). The ^{13}C-NMR. shifts were assigned to corresponding C-atoms by applying the following techniques: a) Proton noise decoupling (PND.) and single frequency off-resonance decoupling (SFORD.); b) comparison with spectra of related compounds. – MS.: *Varian* CH-5 mass spectrometer (ionizing energy 70 eV). The intensity of the molecular ion (M^+) and of the most intense fragment ions is given in % of the base peak (= 100%). Same apparatus was used for GC.-MS. coupling experiments, connected to a *Finnigan*-Incos data system.

1. *Fermentations.* The fungus imperfectus *Lasiodiplodia theobromae* ATCC 28570 originates from the American Type Culture Collection. The β-ionone fermentation is described in details elsewhere [12]. Fermentations were run in a 75 l fermenter equipped with an axial flow impeller with draft tube from *Bioengineering*, Rüti. The working volume of 45 l growth medium contained 5% glucose, 1% cornsteep liquor, 0.05 KCl, 0.1% KH_2PO_4 and 0.1% $NaNO_3$ in tape water. The pH was 6.2 to 6.5 before sterilization. The growth temperature was 28° and the aeration 20 l/min. For transformation 420 g β-ionone were added over 4 days. For small transformation experiments shake cultures were employed of 200 ml same culture medium in 1 l conical flasks with 4 baffles, closed with steel caps. The cultures were shaken in a climatized room at 28° on a rotation shaker at 150 rpm.

2. *Detection of metabolites.* The progress of biotransformation was checked by TLC. and by GC. This was done by withdrawing 50 ml samples of the fermenter broth, extracting them with 50 ml CH_2Cl_2, separating the phases by centrifugation, drying the CH_2Cl_2-phase over Na_2SO_4 and evaporating the solvent under vacuum. TLC. was performed by spotting a solution of the sample onto a silicagel plate *(Merck 60F-254)* and developing with hexane/ethyl acetate 6:4. The metabolites were detected by spraying the plates with acetic acid/H_2SO_4/anisaldehyde 100:2:1, and heating them with hot air. GC. was carried out on a *Carlo Erba Fractovap G1*, using a 3 mm × 2 m glass column with OV-1, 5% on *Gaschrom Q* (60–80 mesh).

3. *Extraction of β-ionone metabolites.* The final extraction of metabolites from the fermenter broth was achieved by employing a centrifugal separator (*Gyrotester* from *Alpha-Laval*) using CH_2Cl_2 or ethyl acetate as extraction solvent. The fermenter broth of *ca.* 45 l was stirred with 30 l CH_2Cl_2 and the phases separated by passing the emulsion through the *Gyrotester*. This procedure was repeated with the water phase. After combining the extracts and drying over Na_2SO_4 and evaporating the solvent under vacuum about 400 g of the brownish β-ionone-transformation product was obtained with a pleasant odor resembling tobacco flavor.

4. *Biotransformations with* Lasiodiplodia theobromae. – a) *Biotransformation of β-cyclo-homogeraniol* (**8**). To 200 ml pre-grown mycelia of *Lasiodiplodia theobromae* ATCC 28570, 500 mg of **8** in 2 ml ethanol were added and incubated under shaking (150 rpm) at 28° for 72 h. The formed metabolites were extracted by addition of 300 ml CH_2Cl_2 to the fermentation broth, followed by shaking and then centrifugation of the emulsion to separate phases. The separated CH_2Cl_2-phase was dried over Na_2SO_4 and the solvent evaporated under vacuum. The remaining oil (380 mg) was analyzed by GC.-MS. The single compounds were identified by comparing their spectra with authentic ones.

b) *Biotransformation of enolacetate* **26**. The biotransformation of **26** was performed in analogy to **8**. The isolated metabolite mixture was analyzed by GC.-MS.

c) *Biotransformation of β-cyclo-homocitral* (**27**) was performed in analogy to **8** and the isolated metabolite mixture was analyzed by GC.-MS.

5. *Synthesis of 3-hydroxy-β-cyclo-homogeraniol* (**17**). – *Synthesis of 1-ethynyl-1,4-dihydroxy-2,2,6-trimethyl-cyclohexane* (**23**). Acetylene was bubbled through a suspension of 5.5 g sodium amide in 60 ml of DMF at 0° for 2½ h. Then at 0° a solution of 6 g of 4-hydroxy-2,2,6-trimethyl-cyclohexanone (**22**) was added, and the mixture stirred overnight at 0°. The mixture was poured onto ice and extracted with ether. The organic phase was washed successively with 2N HCl and water, dried on Na_2SO_4 and evaporated. The residue (3 g) consisting of almost pure compound **23** was recrystallized from ether/pentane, m.p. 112–113°. – IR. ($CHCl_3$): 3650, 3350, 1460, 1080, 1030, 940. – ^{1}H-NMR. (60 MHz): 1.03 (3 H); 1.13 (*s*, 6 H); 3.5–4.3 (*m*, 1 H). – MS.: 164 (2, M^+), 149 (4), 140 (2), 126 (13), 111 (4), 96 (30), 83 (10), 82 (100), 67 (6), 41 (21).

Synthesis of 3-hydroxy-β-cyclo-homocitral (**24**). A solution of 5 g of **23** and 2.2 g triphenyl silanol in 140 ml of xylol was heated to 140°, then 0.5 g of vanadyl isopropylate was added. The mixture was stirred at 130–140° for 12 h, then allowed to reach r.t. After evaporation of the solvent in vacuum the crude mixture was chromatographed on 240 g silicagel *(Merck 60)*. With hexane/ether 4:1 an unknown compound was first eluted, followed by 2.7 g of **24** with hexane/ether 1:1 as eluent. – IR. (liq.): 3400, 2750, 1720, 1470, 1360, 1120, 1040. – ^{1}H-NMR. (400 MHz): 1.01 (*s*, 2 H_3C–C(1)); 1.50 (*t*, $J(H_{ax}$–C(2), H_{ax}–C(3)) = 12, $J(H_{ax}$–C(2), H_{eq}–C(2)) = 12, H_{ax}–C(2)); 1.60 (*s*, H_3C–C(5)); 1.79 (*d* × *qa*, $J(H_{ax}$–C(2), H_{eq}–C(2)) = 12, $J(H_{eq}$–C(2), H_{ax}–C(3)) = 4, $J(H_{eq}$–C(2), H_{eq}–C(4)) = 2, H_{eq}–C(2)); 2.08 (*qa* × *d*, $J(H_{ax}$–C(4), H_{eq}–C(4)) = 16, $J(H_{ax}$–C(4), H_{ax}–C(3)) = 9.5, H_{ax}–C(4)); 2.35 (*qa*, $J(H_{eq}$–C(4), H_{ax}–C(4)) = 16, $J(H_{eq}$–C(4), H_{eq}–C(3)) = 6); 3.10 (*t* large, *J* = 18, 2 H–C(7)); 3.95–4.04 (*m*, H–C(3)); 9.5 (*t*, *J* = 2.4, H–C(8)). – ^{13}C-NMR.: 20.06 (*qa*, C(11)); 27.8 (*qa*, C(9) or C(10)); 29.3 (*qa*, C(9) or C(10)); 37.2 (*s*, C(1)); 42 (1) (*t*, C(4)); 43.6 (*t*, C(7)); 47.8 (*t*, C(11)); 64.4 (*d*, C(3)); 128.4 (C(5) or C(6)); 129.5 (C(6) or C(5)); 200.15 (C(8)). – MS.: 182 (39, M^+), 167 (59), 149 (33), 121 (100), 107 (39), 93 (33), 77 (33), 67 (26), 55 (26), 41 (39).

Synthesis of 3-hydroxy-β-cyclo-homogeraniol (**17**). A solution of 1 g of **24** in 5 ml ether was added to a suspension of 0.3 g $LiAlH_4$ in 40 ml of ether. The mixture was stirred 2 h at r.t., then poured onto a satd. NH_4Cl-solution, extracted with ether, washed with water and dried (Na_2SO_4). After evaporation of the ether the residue was recrystallized from ether/hexane to yield 200 mg of diol **17**, m.p. 124–125°. – IR. ($CHCl_3$): 3670, 3450, 1035. – ^{1}H-NMR. (360 MHz): 1.03 (*s*, H_3C–C(1)); 1.06 (*s*, H_3C–C(1)); 1.41 (*t*, $J(H_{ax}$–C(2), H_{ax}–C(3)) = 12, $J(H_{ax}$–C(2), H_{eq}–C(2)) = 12, H_{ax}–C(2)); 1.66 (*s*, H_3C–C(5)); 1.72 (*d* of *qa*, *J* (H_{ax}–C(2), H_{eq}–C(2)) = 12, $J(H_{eq}$–C(2), H_{ax}–C(3)) = 4, $J(H_{eq}$–C(2), H_{eq}–C(4)) = 2, H_{eq}–C(2)); 1.96 (*qa*, $J(H_{ax}$–C(4), H_{eq}–C(4)) = 16, $J(H_{ax}$–C(4), H_{ax}–C(3)) = 9.5, H_{ax}–C(4)); 2.22–2.44 (*m*, 3 H); 3.52–3.66 (*m*, 2 H–C(8)); 3.87–3.88 (*m*, 2 H–C(3)). – ^{13}C-NMR. (in $CDCl_3$ low solubility, signals weak): 20.8, 28.5, 29.6, 31.8, 42.3, 48.4, 62.3, 65.0, 126.5, 132.8. – ^{13}C-NMR. ($CDCl_3$/DMSO): 19.7 (*qa*, C(11)); 28.2 (*qa*, C(9) or C(10)); 29.6 (*qa*, C(10) or C(9)); 37.0 (*s*, C(1)); 42 (*t*, C(4)); 48.3 (*t*, C(2)); 60.9 (*t*, C(8)); 63.2 (*d*, C(3)); 125.6 (*s*, C(5)); 132.8 (*s*, C(6)). – MS.: 184 (9, M^+), 169 (6), 151 (22), 136 (45), 133 (46), 121 (91), 107 (100), 93 (65), 81 (52), 67 (62), 55 (49), 41 (62).

6. *Chromatography of β-ionone transformation product.* A 60 g sample of crude β-ionone transformation product (total 395 g) obtained from a fermentation run of 420 g β-ionone in a 75 l fermenter containing 45 l culture broth of *Lasiodiplodia theobromae*, was chromatographed on 800 g silicagel *Merck 60* (particle size 0.2–0.5 mm) in a glass column (1.5·0.1 m). By eluting step-wise with 5, 10, 20, 30 and 40% ether in hexane, 4.3 g of not further identified products were obtained. Further elution with 40% ether in hexane yielded 2 g of β-cyclo-homogeraniol (**8**).

Elution with 60% ether in hexane yielded 5 g of a mixture of not further identified products. With 60% and then with 80% ether in hexane 8.6 g of oxo-β-cyclo-homogeraniol (**12**) were eluted. Further elution with 90 to 100% ether yielded 16 g of a mixture of 3-hydroxy- (**17**) and 2-hydroxy-cyclo-homogeraniol (**16**). Further elution with ether and then with ethyl acetate yielded 7.9 g of 4-hydroxy-β-cyclo-homogeraniol (**10**) containing some 2-hydroxy-4-oxo-7,8-dihydro-β-ionone (**19**). Recrystallization from ether/hexane yielded pure diol **10**, m.p. 96–97°. The mother liquor was evaporated to dryness and a 200 mg sample thereof was rechromatographed on 20 g silicagel (*Merck 60*, grain size 0.04 × 0.063 mm). Elution with ethyl acetate/CH_2Cl_2 4:6 yielded 96 mg of **19**, after recrystallization from diisopropyl ether, m.p. 90–92°.

Diols **16** and **17** were subsequently separated by rechromatography of a 200 mg sample of their mixture obtained above on 20 g silicagel (*Merck 60*, particle size 0.04–0.063 mm). Elution with ethyl acetate/CH_2Cl_2 1:9 yielded in a pure fraction 22 mg of 3-hydroxy-β-cyclo-homogeraniol (**17**), m.p. 124–125°, and then 26 mg 2-hydroxy-β-cyclo-homogeraniol (**16**), m.p. 102–104°.

7. *Treatment of 4-hydroxy-β-cyclo-homogeraniol* (**10**) *with potassium hydrogen sulfate.* A solution of 600 mg of the diol **10** and 20 mg hydroquinone in 15 ml of DMSO was heated to 180°. Then 50 mg of $KHSO_4$ were added, and the mixture heated for 15 min, and allowed to reach r.t., then poured in water and extracted with ether. After drying (Na_2SO_4) and evaporation of the solvent the remaining oil was chromatographed by prep. TLC. The solvent system was ether/hexane 1:1. Isolated were 115 mg of 3,4-didehydro-β-cyclo-homogeraniol (**13**) and 8 mg of the bicyclic ether **15**.

Data of 3,4-didehydro-β-cyclo-homogeraniol (**13**). – IR. (liq.): 3400, 1360, 1040. – ^{1}H-NMR. (100 MHz): 1 (*s*, 2 H_3C–C(1)); 1.76 (*s*, H_3C–C(5)); 2.02 (*d*, *J* = 2.5, 2 H–C(2)); 2.44 (*t*, *J* = 8.5, 2 H–C(7)); 3.66 (*d*, *J* = 8.5, 2 H–C(8)); 5.56–5.86 (*m*, olef. H). – ^{13}C-NMR.: 18.4 (*qa*, C(11)); 26.2 (*qa*, C(10) and C(9)); 31.9 (*t*, C(7)); 34.0 (*s*, C(1)); 39.8 (*t*, C(2)); 61.7 (*t*, C(8)); 123.7 (*d*, C(3) or

C(4)); 126.9 (*s*, C(5) or C(6)); 129.1 (*d*, C(4) or C(3)); 134.8 (*s*, C(6) or C(5)). – MS.: 166 (35, M^+), 151 (3), 133 (10), 121 (45), 107 (28), 105 (30), 91 (20), 79 (8), 77 (8), 65 (3), 55 (3), 53 (3), 41 (6), 31 (4).

Data of 3,4-didehydro-β-cyclo-homogeraniol acetate. – UV. (EtOH): 262 (4160). – IR. (liq.): 1740, 1380, 1360, 1240, 1040. – ^{1}H-NMR. (360 MHz): 1 (*s*, 2 H_3C–C(1)); 1.75 (*s*, H_3C–C(5)); 2.03 (*qa*, $J(2,4)=1.4$, $J(3,2)=4$, 2 H–C(2)); 2.05 (*s*, CH_3CO); 2.45 (*t*, $J=8$ and 8, 2 H–C(7)); 4.06 (*t*, $J=8$ and 8, 2 H–C(8)); 5.66 (*d* of *t*, ABX_2, $J(3,2)=4$, $J(3,4)=9$, H–C(3)); 5.73 (*d* of *t*, ABX_2, $J(3,4)=9$, $J(4,2)=1.4$, $J(4,2)=1.4$. – MS.: 208 (3, M^+), 148 (23), 123 (100), 121 (15), 105 (26), 91 (18), 77 (11), 65 (5), 55 (8), 43 (42), 27 (5).

Data of 1,5,5-trimethyl-9-oxa-bicyclo[4.3.0]non-2-en (**15**). – IR. (liq.): 1370, 1100, 1060, 730. – ^{1}H-NMR. (100 MHz): 0.96 (*s*, H_3C–C(5)); 1.02 (*s*, H_3C–C(5)); 1.37 (*s*, H_3C–C(1)); 1.5–2.15 (*m*, 5 H, 2 H–C(7), 2 H–C(8) and H–C(6)); 3.6–3.8 (*m* centered at 3.72, 2 H–C(4)); 5.36–5.74 (*m* centered at 5.56, H–C(3) and H–C(4)). – MS.: 166 (8, M^+), 151 (100), 133 (4), 123 (5), 121 (5), 110 (10), 107 (15), 95 (6), 93 (11), 91 (10), 83 (25), 69 (8), 67 (6), 55 (23), 43 (14), 41 (12).

8. *Physical data of isolated compounds.* – *Data of β-cyclo-homogeraniol* (**8**). – IR. (KBr): 3800, 1450, 1040. – ^{1}H-NMR. (60 MHz): 1.06 (*s*, 2 H_3C–C(1)); 1.7 (*s*, H_3C(5)); 2.4 (*t*, $J=7$, 2 H–C(7)); 3.66 (*t*, $J=7$, 2 H–C(8)). – ^{13}C-NMR.: 19.5 (*s*, C(3)); 20 (*qa*, C(11)); 28.5 (*s*, C(9) and C(10)); 32.2 (*t*, C(7)); 32.8 (*t*, C(4)); 34.6 (*s*, C(1)); 39.8 (*t*, C(2)); 128.8 (*s*, C(6)); 132.9 (*s*, C(5)). – MS.: 168 (40, M^+), 153 (85), 137 (22), 135 (35), 123 (65), 109 (100), 107 (62), 95 (54), 93 (65), 81 (62), 79 (39), 69 (30), 67 (37), 55 (36), 43 (35), 41 (60).

Data of β-cyclo-homogeraniol acetate (**9**). – IR. (liq.): 2950, 1740, 1380, 1360, 1240, 1150, 970. – ^{1}H-NMR. (60 MHz): 1,03 (*s*, 2 H_3C–C(1)); 1,66 (*s*, H_3C–C(5)); 2.06 (*s*, CH_3CO); 2.36 (*t*, $J=9$, 2 H–C(7)). – ^{13}C-NMR.: 19.5 (*t*, C(3)); 19.9 (*qa*, C(11)); 20.6 (*qa*, CH_3CO); 28.0 (*t*, C(7)); 28.4 (2 *qa*, C(9) and C(10)); 32.8 (*t*, C(4)); 34.6 (*s*, C(1)); 39.7 (*t*, C(2)); 63.5 (*t*, C(8)); 129.8 (*s*, C(5)); 132.3 (*s*, C(6)); 169.9 (*s*, C=O). – MS.: 210 (6, M^+), 150 (48), 135 (100), 123 (6), 121 (6), 107 (20), 93 (18), 79 (18), 69 (4), 67 (4), 55 (5), 43 (15).

Data of 4-oxo-β-cyclo-homogeraniol (**12**). – UV. (EtOH): 248 (12600). – IR. (liq.): 3450, 1660, 1601, 1200, 1040. – ^{1}H-NMR. (360 MHz): 1.17 (*s*, 2 H_3C–C(1)); 1.8 (*s*, H_3C–C(5)); 1.8 (*t*, $J=6.5$, 2 H–C(2)); 2.46 (*t*, $J=6.5$, 2 H–C(3)); 2.58 (*t*, $J=8$, 2 H–C(7)); 3.73 (*t*, $J=8$, 2 H–C(8)). – ^{13}C-NMR.: 11.8 (*qa*, C(11)); 26.7 (*qa*, C(9) and C(10)); 34.1 (*t*, C(2) or C(3) or C(7)); 34.4 (*t*, C(7) or C(2) or C(3)); 36.1 (*s*, C(1)); 37.1 (*t*, C(3) or C(7) or C(2)); 6.05 (*t*, C(8)); 131.8 (*s*, C(5) or C(6)); 161.2 (*s*, C(6) or C(5)); 199.1 (*s*, C(4)). – MS.: 182 (4, M^+), 167 (100), 149 (17), 137 (40), 126 (55), 109 (48), 93 (24), 81 (23), 67 (25), 55 (19), 41 (20).

Data of 4-hydroxy-β-cyclo-homogeraniol (**10**). – IR. (KBr): 3400, 1350, 1040. – ^{1}H-NMR. (100 MHz): 0.98 (*s*, H_3C–C(1)); 1.05 (*s*, H_3C–C(1)); 1.78 (*s*, H_3C–C(5)); 2.34 (*t*, $J=8$, 2 H–C(7)); 3.65 (*t*, $J=8$, H–C(8)); 3.94 (*t*, $J=4$, H–C(4)). – ^{13}C-NMR.: 17.3 (*qa*, C(11)); 27.2 (*qa*, C(9) or C(10)); 28.5 (*qa*, C(10) or C(9)); 28.6 (*t*, C(3)); 32.2 (*t*, C(7)); 34.6 (*t*, C(2)); 35.1 (*s*, C(1)); 61.5 (*t*, C(8)); 69.7 (*d*, C(4)); 130.8 (*s*, C(5) or C(6)); 137.5 (*s*, C(6) or C(5)). – MS.: 184 (28, M^+), 169 (12), 151 (9), 139 (100), 128 (71), 121 (27), 109 (30), 95 (30), 81 (18), 72 (22), 55 (15), 43 (40).

Data of 2-hydroxy-β-cyclo-homogeraniol (**16**). – IR. (KBr): 3300, 1320, 1360, 1040, 1020. – ^{1}H-NMR. (360 MHz): 1.02 (*s*, H_3C–C(1)); 1.07 (*s*, H_3C–C(1)); 1.65 (*s*, H_3C–C(5)); 2.05 (*m*, 2 H–C(4)); 2.36 (*t*, $J=8$, 2 H–C(7)); 3.49 (*qa*, $J(H_{ax}$–C(2), H_{ax}–C(3))$=9$, $J(H_{ax}$–C(2), H_{eq}–C(3))$=3$, H–C(2)); 3.61 (*t*, $J=8$, 2 H–C(8)). – MS.: 184 (11, M^+), 166 (16), 151 (10), 136 (44), 121 (100), 107 (77), 97 (37), 93 (45), 81 (49), 67 (49), 55 (40), 43 (72), 31 (43).

Data of 2-hydroxy-β-cyclo-homogeraniol acetate. – ^{1}H-NMR. (360 MHz): 1.01 (*s*, H_3C–C(1)); 1.07 (*s*, H_3C–C(1)); 1.66 (*s*, H_3C–C(11)); 2.04 (*s*, CH_3CO); 2.05 (*t*, $J=8$, 2 H–C(4)); 2.38 (*t*, $J=7$, 2 H–C(7)); 3.49 (*qa*, $J(H_{ax}$–C(2), H_{ax}–C(3))$=9$, $J(H_{ax}$–C(2), H_{eq}–C(3))$=3$, H–C(2)); 3.96–4.09 (*m*, centered at 4.02, 2 H–C(8)).

Data of 2-hydroxy-4-oxo-7,8-dihydro-β-ionone (**19**). – UV. (EtOH): 246 (13800). – IR. (KBr): 3400, 1715, 1660, 1620, 1420, 1365, 1330, 1170, 1060, 1005. – ^{1}H-NMR. (360 MHz): 1.17 (*s*, H_3C–C(1)); 1.22 (*s*, H_3C–C(1)); 1.75 (*s*, H_3C–C(5)); 2.19 (*s*, 3 H–C(10)); 2.5–2.63 (*m*, 2 H–C(7), 2 H–C(8) and H_{ax}–C(3)); 2.66 (ABX-system, $J(H_{ax}$–C(3), H_{eq}–C(3))$=17$, $J(H_{ax}$–C(3), H_{ax}–C(2))$=9$, H_{eq}–C(2)); 2.75 (ABX-system, $J(H_{eq}$–C(3), H_{ax}–C(2))$=4.5$, $J(H_{ax}$–C(3), H_{eq}–C(3))$=17$, H_{eq}–C(3)); 3.84 (*qa*, $J(H_{eq}$–C(3), H_{ax}–C(2))$=4.5$, $J(H_{ax}$–C(2), H_{ax}–C(3))$=9$, H_{ax}–C(2)). – ^{13}C-NMR.: 11.3 (*qa*, C(13)); 20.6 (*qa*, C(12) or C(11)); 24.0 (*t*, C(17)); 24.7 (*qa*, C(11) or C(12)); 29.6

(*qa*, C(10)); 41.3 (*t*, C(8) or C(3)); 42.1 (*s*, C(1)); 42.5 (*t*, C(3) or C(8)); 73.4 (*d*, C(2)); 130.9 (*s*, C(5) or C(6)); 162 (*s*, C(6) or C(5)); 197 (*s*, C(4) or C(9)); 206 (*s*, C(9) or C(4)). – MS.: 206 (49, M^+), 188 (16), 173 (30), 163 (36), 148 (100), 135 (52), 119 (10), 115 (9), 105 (19), 91 (18), 77 (10), 43 (27).

Data of 2-acetoxy-4-oxo-7,8-dihydro-β-ionone. – IR. (liq.): 1715, 1660, 1620, 1240, 1170, 840. – ^{1}H-NMR.: 1.15 (*s*, $H_3C(11)$ or $H_3C(12)$); 1.18 (*s*, $H_3C(12)$ or $H_3C(11)$); 1.76 (*s*, $H_3C-C(5)$); 2.05 (*s*, $OCOCH_3$); 2.19 (*s*, $H_3C(10)$); 2.46–2.63 (*m*, 2 H–C(7), 2 H–C(8) and H_{ax}–C(3)); 2.58 (*A*B*X*-system, $J(H_{ax}-C(3), H_{ax}-C(2)) = 8$, $J(H_{ax}-C(3), H_{eq}-C(3)) = 17$); 2.77 (A*B*X-system, $J(H_{eq}-C(3), H_{ax}-C(2)) = 4.5$, $J(H_{eq}-C(3), H_{ax}-C(3)) = 17$, H_{eq}–C(3)); 5.05 (*qa*, $J(H_{ax}-C(2), H_{ax}-C(3)) = 8$, $J(H_{ax}-C(2), H_{eq}-C(3)) = 4.5$, H_{ax}–C(2)). – MS.: 266 (14, M^+), 254 (10), 223 (14), 206 (30), 188 (28), 173 (30), 163 (95), 148 (44), 135 (100), 121 (60), 105 (80), 91 (64), 79 (48), 67 (30).

Data of 4-oxo-2,3-didehydro-7,8-dihydro-β-ionone (**21**). – UV. (EtOH): 237 (8540). – IR. ($CHCl_3$): 1710, 1660, 1620, 1400, 1330, 1160, 1000, 840. – ^{1}H-NMR. (360 MHz): 1.23 (*s*, 2 $H_3C-C(1)$); 1.86 (*s*, $H_3C-C(5)$); 2.19 (*s*, 3 H–C(10)); 2.60 (*s*, 2 H–C(7) and 2 H–C(8)); 6.19 (AB-system, $J(2,3) = 10$, H–C(3)); 6.73 (AB-system, $J(2,3) = 10$, H–C(2)). – MS.: 206 (21, M^+), 188 (16), 173 (29), 163 (69), 149 (62), 135 (90), 121 (40), 105 (100), 91 (60), 79 (32), 71 (27), 57 (30).

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