125. Biosynthesis of Dodecano-4-lactone in Ripening Fruits: Crucial Role of an Epoxide-Hydrolase in Enantioselective Generation of Aroma Components of the Nectarine (*Prunus persica* var. *nucipersica*) and the Strawberry (*Fragaria ananassa*)

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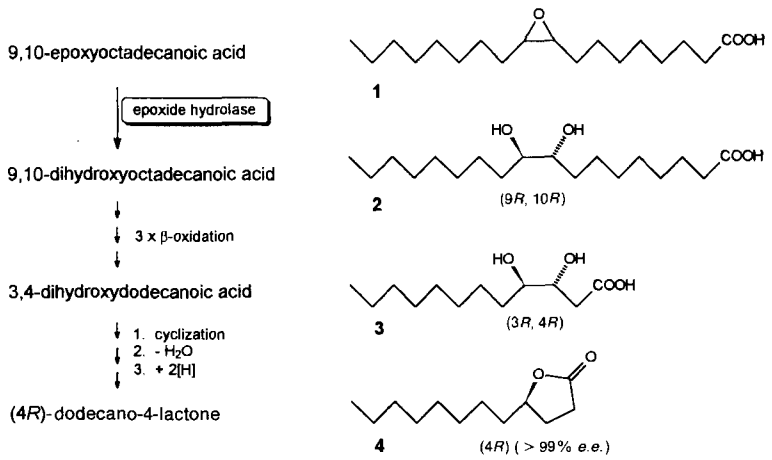
Early steps in the biosynthesis of dodecano-4-lactone in ripening nectarines (*Prunus persica* var. *nucipersica*) and strawberries (*Fragaria ananassa*) were studied using *cis*-9,10-([^{18}O]epoxy)heptadecanoic acid (**5**), instead of the natural C_{18}-precursor epoxystearic acid **1**. In nectarines, an epoxide hydrolase catalyzed, in a highly regio- and enantioselective reaction, the attack of H_2O at C(9) of epoxy acid **5** yielding preferentially (9*R*,10*R*)-dihydroxyheptadecanoic acid **6a** as an early intermediate and, after further metabolization, undecano-4-lactone **7a**. In strawberries, the same transformation exhibited only a moderate regio- and enantioselectivity. Besides the previously established function of phytogenic epoxide hydrolases involved in generating oxygenated fatty-acid derivatives, which are used in cutin monomer production and plant defence, a new rôle for these enzymes now emerges in the enantioselective production of aroma components of ripening fruits. The *cis*-9,10-([^{18}O]epoxy)heptadecanoic acid ($\geq$ 98% ^{18}O; **5**) was obtained in good overall yield (68%) from isopropyl (9*Z*)-heptadec-9-enoate using $^{18}O_2$ gas in a free-radical chain reaction in the presence of 2-methylbutanal.

Introduction. – δ- and γ-lactones with six to twelve C-atoms are ubiquitous in nature and have been isolated from bacterial, plant, and insect sources [1–3]. Even higher animals use lactones, but here the macrocyclic, musk-type odourants predominate [4]. Fruits, a particularly rich source of lactones, often emit the γ-lactones decano-4-lactone or dodecano-4-lactone, or a mixture of the two, in order to attract feeders for seed dispersal. From recent biosynthetic studies, it has become clear that several enzymatic routes, generally starting from unsaturated fatty acids, are responsible for the structural diversity of this class of natural products [5]. The introduction of O-atoms is generally achieved by *i*) hydratization of double bonds (*e.g.* linoleic acid → ricinoleic acid), *ii*) direct functionalization of nonactivated CH_2 groups by mixed-function oxygenases, and *iii*) introduction of a hydroperoxide into a pentadienyl segment of linoleic- and linolenic acid by a lipoxygenase [6] [7]. Recently, we have shown that dodecano-4-lactone (**4**), at least, is produced by a different route. In this case, oleic acid, or a derivative, is functionalized by epoxidation yielding 9,10-epoxystearic acid (**1**) as an early intermediate *en route* to **4** (*Scheme 1*) [8].

The novel pathway (*Scheme 1*) relies on enzymatic reactions which are ubiquitous in the plant kingdom and which are often involved in plant defence against microbial aggressors. Both (9*R*,10*S*)-configurated epoxystearic acid **1** and its derivative (9*R*,10*R*)-dihydroxy acid **2** (with the essential (*R*)-configuration at C(10) to produce (4*R*)-configurated **4** *via* **3**) are widely found in higher plants and seed oils [9] [10]. Accordingly, administration of D-labelled 9,10-epoxyheptadecanoic acid **5** and 9,10-dihydroxyhep-

Scheme 1. *Proposed Biosynthesis of Dodecano-4-lactone* (**4**) *in Ripening Fruits.* The sequence was previously established by administration of D-labelled precursors of type **5** and **6** to ripening strawberries and peaches [8].

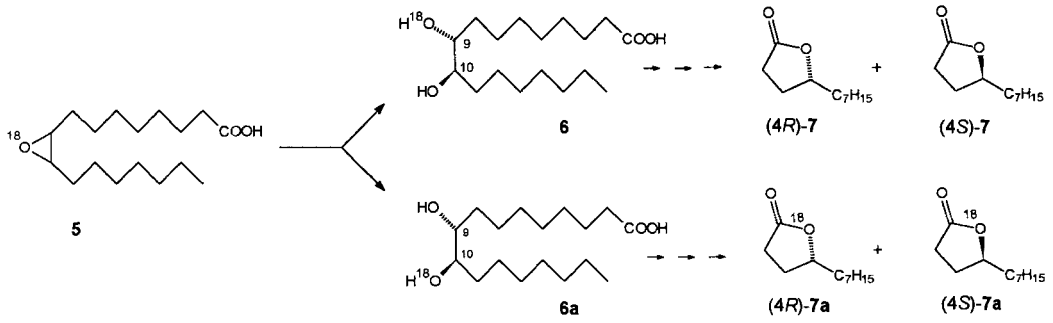


tadecanoic acid **6** (lower homologues of the natural C_{18}-precursors) resulted in production of (4*R*)-undecano-4-lactone (4*R*)-**7** by strawberries and peaches [8]. Thus, the pathways of plant defence and odour production in ripening fruits appear to have some transformations in common. The type of enzymes required for the production and transformation of the early precursors in *Scheme 1*, namely a fatty-acid peroxygenase and an epoxide hydrolase (EC 3.3.2.3), have been recently isolated from soybean (*Glycine max*). Careful mechanistic studies by *Blée* and *Schuber* revealed some unique properties of the phytogenic enzymes which are remarkably different [11] [12] from the well studied epoxide hydrolases from animal sources [13] [14], which are used in general detoxification [15]. The soybean enzyme converts both enantiomers of 9,10-epoxystearic acid **1** into (9*R*,10*R*)-dihydroxy acid **2** (*ca.* 90% ee). By using [^{18}O]-**1**, it was shown that the enzyme catalyzes the addition of H_2O almost exclusively to that C-atom at the oxirane ring which possess (*S*)-chirality, the (9*R*,10*S*)-enantiomer being kinetically preferred.

It will be shown, in this work, that an enzyme, similar to the epoxide hydrolase from soybeans, is involved in the biosynthesis of dodecano-4-lactone (**4**) in ripening fruits. In nectarines (*Prunus persica* var. *nucipersica*), the configuration of the resulting dihydroxy acid **2** (*cf. Scheme 1* and **6**/**6a** in *Scheme 2*) resembles that of the acid produced by the soybean system [11], but the regioselectivity is opposite.

2. Synthesis of *cis* 9,10-([^{18}O]Epoxy)heptadecanoic Acid (5). – To test for the involvement of an epoxide hydrolase in the biosynthesis of dodecano-4-lactone (**4**), the mode of hydrolysis of *cis*-9,10-([^{18}O]epoxy)heptadecanoic acid (**5**) to the two isomeric 9,10-[^{18}O]-dihydroxy acids **6**/**6a** was studied. As outlined in *Scheme 2*, the retention or loss of ^{18}O in the lactone product should reflect the regioselectivity of hydrolysis, while the optical purity of **7**/**7a** should largely correspond to the degree of enantioselectivity of the epoxide hydrolase.

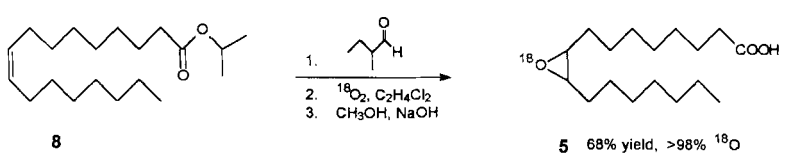
Scheme 2. *Pathway of the [^{18}O]-Label from* **5** *into Undecano-4-lactones* **7**/**7a**. According to the regioselectivity of the epoxide hydrolase, the [^{18}O]-label was found at C(9) or C(10) of the resulting dihydroxy acid **6**/**6a**.



The latter conclusion is justified by our previous observation that (in peaches) the incorporation of racemic dihydroxy acid **6** results in undecano-4-lactone **7** of only low ee (14%), while the racemic epoxy acid yielded undecano-4-lactone of 58% ee, indicating that the enzymes of the later stages of lactone biosynthesis (those stages after epoxide hydrolysis) do not significantly enhance the optical purity of the intermediates *en route* to **7**. The use of the unnatural C_{17}-precursor **5** is essential to avoid the masking of metabolites by natural products, which interfere with the determination of optical purities and isotope ratios [8].

[^{18}O]-Labelled epoxides have been prepared from olefins and molecular oxygen in the presence of transition-metal catalysts [16] or by peroxygenase-mediated O-transfer from 13-([^{18}O]hydroperoxy)linoleic acid [17]. Both methods utilize readily available $^{18}O_2$ gas, but suffer from tedious catalyst preparation or low incorporation of expensive $^{18}O_2$ [17]. Recently, *Kaneda* reported a facile epoxidation of olefins with molecular oxygen and an aldehyde, preferably 2-methylbutanal, as the co-substrate in a free-radical chain reaction [18] (*cf. Scheme 3*).

Scheme 3



Application of the *Kaneda* conditions to isopropyl (9*Z*)-heptadec-9-enoate (**8**) [8] resulted in a fast and highly effective incorporation of $^{18}O_2$ (using 99% $^{18}O_2$) into the substrate. Saponification of the intermediate epoxy ester yielded the free acid **5** in good yield (90%). According to the mass spectroscopic analysis of the relevant fragment of **5** at *m*/*z* 187/185, corresponding to $[[^{18}O/^{16}O]M - (CH_2)_6Me]^+$, the isotope ratio $^{18}O/^{16}O$ is *ca.* 98:2. Furthermore, the pure epoxy acid **5** was obtained in 68% overall yield, thus

underlining the efficiency and simplicity of the *Kaneda* protocol for the synthesis of [^{18}O]-labelled epoxides.

3. Incubation Experiments and Analysis of Volatiles. – The isotopically labelled epoxy acid **5**, dissolved in H_2O (0.4% solution) by sonication together with *Triton® X 100*, was injected subepidermally into ripening nectarines (cv. *May Glo*, South Africa) and strawberries (cv. *Elsanta*, Belgium). Painting the fruits with a soln. of precursor **5** was also performed, but proved to be less effective. Next, the pretreated fruits were placed in a closed system, and the emitted volatiles were continuously collected on a charcoal trap as described previously [19] [20] (*cf. Exper. Part*). The expected isotopomeric undecanolactones **7**/**7a** were released from the nectarines into the gas phase as soon as 6 h after injection of **5**. During the second collection period, 6–12 h after injection, the amount of **7**/**7a** already exceeded the amount of natural decano-4-lactone and dodecano-4-lactone (**4**) by a factor of *ca.* 1.5 (*cf. Fig. 1* (3rd collection period)), and after 24 h, a five-fold excess of **7**/**7a** over **4** was observed.

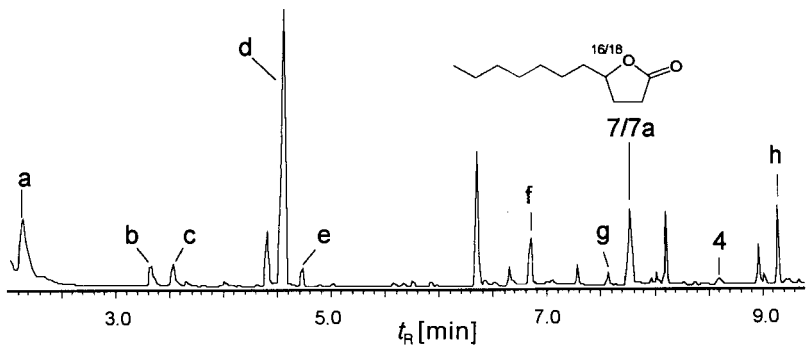


Fig. 1. *Profile of the volatiles from nectarines, collected 12–18 h after incubation (subepidermal injection) with epoxy acid* **5**. Separation of compounds was achieved on a fused silica column coated with *DB 1* (15 m × 0.25 mm) under programmed conditions (50° for 2 min, then to 200° at 15°/min, followed by 20°/min to 280°). Injected amount: 2 µl. Detection and identification of compounds: quadrupole mass spectrometer, *Fisons MD 800*. Scan range: 35–350 Da sec^{-1}, interface at 270°. Identified compounds: (a) (*Z*)-hex-3-enyl acetate, (b) methyl heptanoate, (c) undecane, (d) methyl octanoate, (e) dodecane, (f) decano-4-lactone, (g) pentadecane, **7**/**7a** isotopomers of undecano-4-lactone, **4** dodecano-4-lactone, (h) heptadecane.

As expected, there was complete resolution of **7**/**7a** from the natural lactones and other volatiles of the nectarines by gas chromatography. The mass spectrum of the GLC peak for **7**/**7a** (collected after 12–18 h) showed two major fragments at *m*/*z* 85 (42% rel. int.) and 87 (100% rel. int.), corresponding to the differently labelled dihydrofuran-2-one segment of the undecano-4-lactones **7**/**7a**. Since unlabelled undecano-4-lactone showed a base peak at *m*/*z* 85, without satellites at *m*/*z* 83 and 87, the relative intensity of the two fragments in the mass spectrum of the metabolite directly corresponded to the ratio **7**/**7a** (*cf. Fig. 2*).

Interestingly, the ratio **7**/**7a** was not constant, but was found to vary with the progress of the metabolization of **5**. During the early stages of the incubation experiment (6 h after administration), the fraction of **7** in **7**/**7a**, calculated from the relative intensities of the major fragments at *m*/*z* 85 and 87 (*cf. Fig. 2* and also *Scheme 2*), was *ca.* 23% (*cf. Fig. 3*).

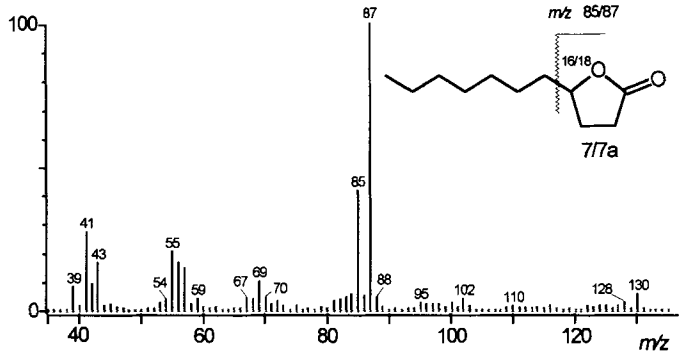


Fig. 2. *Mass spectrum* (70 eV) *of isotopomeric undecano-4-lactones* **7**/**7a**, *from the headspace of nectarines, collected 12–18 h after subepidermal injection of* **5**. Separation and MS conditions, *cf. Fig. 1.*

During the later stages, the fraction of **7** (proportional to the intensity of *m*/*z* 85) steadily increased and reached a final level of *ca.* 38% after about four days. This result suggested H_2O is preferentially attacking C(9) of epoxy acid **5** (*Scheme 2*), placing the ^{18}O-label at C(10) of the substrate yielding **6a** in excess. All subsequent transformations do not involve this C–^{18}O bond and, hence, the label was still present in the undecano-4-lactone **7a**.

While the ratio of **7**/**7a** varied significantly with time, the ee of the metabolites changed only moderately (decrease from 72 to 62% ee within four days; see *Fig. 3*). Baseline separation of (4*R*)- and (4*S*)-undecano-4-lactones **7**/**7a** was readily achieved by GLC using a fused silica column coated with octakis(2,6-di-*O*-pentyl-3-*O*-butyryl)-γ-cyclodextrin (*Lipodex E*®, *Macherey & Nagel*, Düren). Unlike the artificially induced

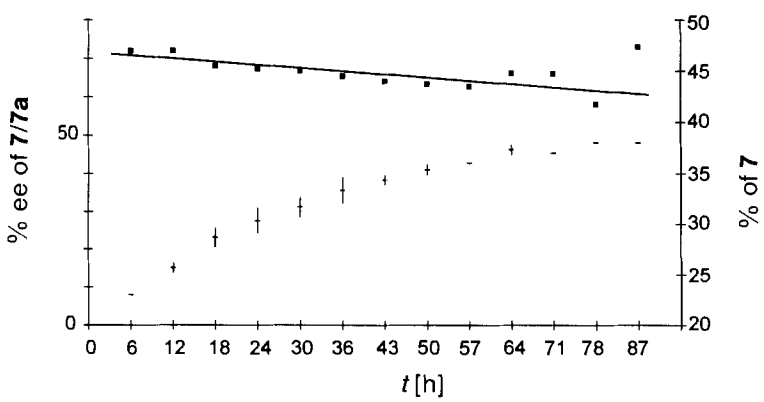


Fig. 3. *Progress curve of the hydrolysis of epoxy acid* **5**. Right axis (–): Relative fraction (%) of **7** in **7**/**7a** as function of time. The relative fraction (%) of **7** is calculated according to the formula $(a/(a + b))100$ (where *a* is the intensity of the ion at *m*/*z* 85 and *b* the intensity of the ion at *m*/*z* 87, *cf. Fig. 2*). Left axis (■): Enantiomeric excess (ee) of undecano-4-lactones **7**/**7a** as the function of time. Volatiles were collected in the intervals given. The chiral analysis was achieved on a fused silica column coated with octakis(2,6-di-*O*-pentyl-3-*O*-butyryl)-γ-cyclodextrin (*Lipodex E*®, *Macherey & Nagel*, Düren) under programmed conditions (130° for 7 min, then 2°/min to 160°).

undecano-4-lactone **7**/**7a**, the optical purities of the natural decano- and dodecano-4-lactones were higher in both fruits ($\geq 88\%$ and $\geq 94\%$ ee, resp.).

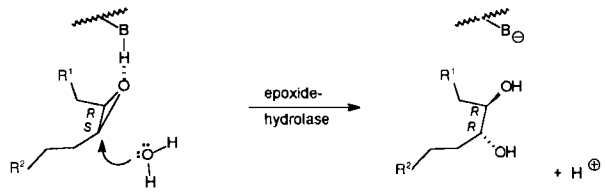
The results of the experiments with strawberries resembled those obtained with nectarines, but the degree of the regio- and enantioselectivity of the enzymes involved was very different. During early stages of the incubation experiments, the relative intensity of the ^{18}O-containing dihydrofuran-2-one fragment (*m/z* 87 (100%)) was significantly higher than that of the ^{16}O-containing fragment at *m/z* 85 (71%), indicating a slightly more favourable attack of H_2O at C(9) of epoxy acid **5**. In the later stages, the ratio $^{18}O/^{16}O$ in the fragments decreased to give finally, after 36 h, a slight preference for addition of H_2O to C(10), as indicated by *m/z* 85 (100%) and *m/z* 87 (93%). Within the same period, the degree of the enantioselectivity for the transformation of **5** to **7**/**7a** dropped from 51 to 44% ee after 36 h.

In an additional experiment, the individual enantiomers of the metabolites were exemplarically checked for their $^{16}O/^{18}O$ ratio as a function of time (strawberry, cv. *Salerno*; Italy). The two enantiomers showed only a marginally different $^{16}O/^{18}O$ ratio ((4*R*)-**7**/(4*R*)-**7a** 1:0.84, (4*S*)-**7**/(4*S*)-**7a** 1:0.75) which remained largely constant within the first 30 h. Later stages (after *ca.* 50 h) exhibited a slightly different isotope composition of both enantiomers ((4*R*)-**7**/(4*R*)-**7a**: 1:0.89, (4*S*)-**7**/(4*S*)-**7a**: 1:0.69).

Incubation experiments with epoxy acid **5** were also carried out with homogenates of the nectarines in a phosphate buffer (pH 7). In this case, besides very small traces of undecano-4-lactone **7a**, the dihydroxy acids **6**/**6a** accumulated, due to ineffective β-oxidation. The identity of the isolated metabolite was secured by comparative GLC and mass spectroscopy with an authentic sample [8] and, thus, provides additional confirmation for the sequence of *Scheme 1*.

4. Discussion. – The results show a remarkable coincidence with data previously reported for an epoxide hydrolase isolated from soybeans [12]. During lactone biosynthesis, as well as in studies with the isolated soybean enzyme, H_2O is added in a *trans*-fashion to epoxy stearic acid **1** yielding *threo*-configurated (9*R*,10*R*)-dihydroxy acid **2**. In fruits, **2** (*Scheme 1*) is further degraded by β-oxidation to give the dihydroxy acid **3**, which, after cyclization, loss of H_2O, and reduction of the resulting α,β-unsaturated lactone yields dodecano-4-lactone (4) (*Scheme 1*) [8]. Both epoxide hydrolases exhibit a characteristic degree of regio- and enantioselectivity. However, in contrast to the soybean enzyme, which catalyses the addition of H_2O to C(10) of 9,10-epoxystearic acid **1**, the epoxide hydrolase from mature nectarines cleaves the C(9)–O bond of **5** selectively and, hence, leads preferentially to the ^{18}O-lactone **7a** (*Scheme 2*). In the early stages of the incubation, the transformation of the racemic precursor **5** into undecano-4-lactone results in a $[^{18}O/^{16}O]$ ratio of 77:23. As the metabolization proceeds, (9*R*,10*S*)-**5** accumulates, and the transformation rate of the less preferred enantiomer slowly increases yielding the unlabelled lactone **7**. It is very important to note, however, that at the same time the degree of the enantioselectivity of the enzyme is not significantly changed. This implies that both enantiomers of **5** are converted into *threo*-configurated (9*R*,10*R*)-dihydroxyheptadecanoic acid. As already pointed out by *Blée* and *Schuber*, this stereochemical outcome is readily explained by assuming that the active site of the enzyme accommodates the substrate in such a fashion that a centre with (*S*) chirality can always be attacked (*Scheme 4*).

Scheme 4



This preference for the (*S*)-configurated centre of either (9*R*,10*S*)-**5** or (9*S*,10*R*)-**5** is readily achieved by rotating either enantiomer by 180° within the active site, relative to a fixed H_2O molecule and an epoxide activating functionality (*e.g.* a proton source). This mechanism explains why the ratio of the differently labelled undecano-4-lactones **7**/**7a** may vary, while their ee is not substantially affected. The orientation of the preferentially hydrolyzed precursor depends to a certain extent on the presence of a free carboxylate group at C(1), since the replacement of –COOH by –COO(i-Pr) lowers the regioselectivity of the epoxide hydrolysis. In the nectarine, however, the effect is much less pronounced than previously reported for the soybean enzyme [11].

As a consequence of our findings, it is now reasonable to assume that epoxidation of unsaturated fatty acids like, *e.g.*, linoleic and linolenic acid may represent a common pathway to oxygenated derivatives of fatty acids which are, in fruits, channelled into production of the corresponding (un)saturated γ- and δ-lactones. The concept is strongly supported by the result of an administration experiment of a mixture of all three monoepoxides of linolenic acid to nectarines. The labelled [^{18}O]-precursors were, indeed, smoothly converted into dodeca-6,9-dieno-4-lactone and hexano-4-lactone, both known as natural products. In the yeast *Sporidiobulus salmonicolor*[1]), 12,13-epoxyoctadec-9-enoic acid (vernolic acid) was effectively transformed into 5-hydroxydecano-4-lactone [21], whilst nectarines metabolize the positional isomer, namely 9,10-epoxyocadec-12-enoic acid into dodec-6-eno-4-lactone. Further experiments into this direction with additional yeasts, fungi, and ornamentals as test organisms were already encouraging and will be reported soon.

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Experimental Part

General. Fruits were bought in local stores in Bonn; the cultivars are denoted in the text. Solvents were dried prior to use. Gas chromatography: *Carlo Erba, Series 4100*, equipped with fused-silica capillaries coated with *SE 30* (10 m × 0.31 mm) or 6-methyl-2,3-di-*O*-pentyl-γ-cyclodextrin (*Lipodex*® *E*, 50 m × 0.31 mm) from *Macherey & Nagel* (D–52313 Düren, Germany). Column chromatography: silica gel *Si 60* (0.2–0.5 mm) from *E. Merck*

[1]) The yeast was previously renamed from *Sporobolomyces odorus* to *Sporidiobolus salmonicolor*.

(D–64271 Darmstadt). Thin-layer chromatography: silica gel *60* F_{254} TLC plates from *Merck*. IR Spectra: *Perkin-Elmer* IR spectrophotometer *1600*. ^{1}H-NMR: in $CDCl_3$ on a *Bruker AM 250*; chemical shifts δ in ppm. GC/MS: *Fisons MD 800* or *Finnigan ITD 800*, equipped with a *SE 30* (15 m)- and *DB-1* (15 m × 0.25 mm)-coated fused-silica capillary column, resp. EI-HR-MS (70 eV): *Kratos-MS-50* instrument.

cis-*9,10-([^{18}O]Epoxy)heptadecanoic Acid* (= cis-*3-Heptyl[^{18}O]oxiran-2-octanoic Acid*; **5**). Through dry dichloroethane (10 ml) in a He-flushed (repeatedly) flask, He was slowly bubbled through the solvent for 15 min to remove dissolved O_2. The solvent was gently heated (40°) and $^{18}O_2$ bubbled through while stirring well. Freshly distilled 2-methylbutanal (0.3 ml, 3.37 mmol) was injected through a silicon-rubber septum, and after 30 min, the isopropyl heptadecenoate (**8**) [8] (0.295 g, 0.95 mmol) in CH_2Cl_2 (2 ml) was introduced. Stirring was continued at 40° with $^{18}O_2$ (99% $^{18}O_2$; *KFK*, Karlsruhe, Germany) continuously bubbling through the soln. Usually, after *ca.* 2 h, more than 90% of the olefinic acid was converted into the 9,10-epoxy ester (GLC monitoring). The org. layer was washed with dil. aq. $NaHSO_3$ (5 ml) and aq. $NaHCO_3$ soln. (5 ml), the aq. phase extracted with Et_2O (2 × 10 ml), and the combined org. layer washed with H_2O and dried (Na_2SO_4). Pure ester (0.233 g, 75%) was obtained by CC (SiO_2, pentane/Et_2O 98:2→95:5 (*v/v*)). The saponification of the epoxy ester was achieved in MeOH/NaOH as described [8]: 0.183 g (90%) of **5**. IR (Film): 3600–2400 (br.), 1693, 1470, 1261, 1227, 1194, 928, 833, 718. ^{1}H-NMR ($CDCl_3$): 2.95–2.83 (*m*, H–C(9), H–C(10)); 2.35 (*t*, 2 H–C(2)); 1.70–1.55 (*q*, 2 H–C(3)); 1.55–1.05 (*m*, 20 H); 0.88 (*t*, 3 H–C(17)). MS (70 eV): 286 (0.3 $M^{+\cdot}$), 268 (0.3), 187 (12), 169 (4), 157 (69), 143 (21), 139 (12), 120 (14), 109 (29), 97 (33), 95 (29), 94 (27), 83 (31), 81 (30), 73 (15), 69 (89), 67 (41), 55 (100); according to the rel. abundance of $M^{+\cdot}$ (*m/z* 286/284) or the more abundant ions $[[^{18}O]M - (CH_2)_6Me]^+$ (*m/z* 187/185), $^{18}O/^{16}O$ > 98:2. HR-MS: 286.2402 ($C_{17}H_{32}{}^{16}O_2{}^{18}O^+$, $M^{+\cdot}$; calc.: 286.2398); 187.1228 ($C_{10}H_{17}{}^{16}O_2{}^{18}O^+$; calc. 187.1224).

Application of Precursors and Analysis of Volatiles. Fruits: Strawberries (*Fragaria ananassa*), cv. *Elsanta*, were from Belgium; strawberries, cv. *Salerno*, were from Italy. Nectarines (*Prunus persica* var. *nucipersica*), cv. *May Glo*, were from South Africa. Other cultivars (*e.g. Armking* from Argentina) proved to be less productive. A suspension of epoxy acid **5** (5.0 mg) in H_2O (2 ml) was sonicated together with 2 drops of *Triton X 100* (*Sigma*). The resulting clear soln. was injected subepidermally into ripening fruits. In a single experiment, *ca.* eight strawberries or two nectarines were used for incubation and odour collection. Only fruits with a clearly discernible fragrance and significant lactone production (GLC) were selected to warrant a comparable degree of maturation. After injection of the precursors, the fruits were transferred into a closed system, and *ca.* 1 h later, collection of volatiles was started by air circulation and adsorption of the released volatiles on a charcoal trap (1.5 mg, *CLSA Filter*, Winterthur, Switzerland) [19] [20]. The pattern of the emitted volatiles was followed over a prolonged period (up to 4 days) by changing the charcoal traps in 6–9 h intervals. Following desorption of the charcoal traps (2 × 20 µl CH_2Cl_2), the volatiles were analyzed by GLC/MS for identification and by GLC on a fused-silica capillary coated with octakis(2,6-di-*O*-pentyl-3-*O*-butyryl)-γ-cyclodextrin (*Lipodex E*®, *Macherey & Nagel*, Düren, Germany) for determination of the enantiomer composition [22]. The identity of compounds was established by comparison with an authentic sample. Each experiment was repeated in triplicate. Data concerning the isotope composition and the enantiomeric excess of the metabolites are given in *Fig. 3*; standard deviations were calculated and are included in the figure.

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