Stereo-Inversion in the (4*R*)-γ-Decanolactone Synthesis by *Saccharomyces cerevisiae:* (2*E*,4*S*)-4-Hydroxydec-2-enoic Acid Acts as a Key Intermediate

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Methyl (2*E*,4*R*)-4-hydroxydec-2-enoate, methyl (2*E*,4*S*)-4-hydroxydec-2-enoate, and ethyl (±)-(2*E*)-4-hydroxy[4-^{2}H]dec-2-enoate were chemically synthesized and incubated in the yeast *Saccharomyces cerevisiae*. Initial C-chain elongation of these substrates to C_{12} and, to a lesser extent, C_{14} fatty acids was observed, followed by γ-decanolactone formation. Metabolic conversion of methyl (2*E*,4*R*)-4-hydroxydec-2-enoate and methyl (2*E*,4*S*)-4-hydroxydec-2-enoate both led to (4*R*)-γ-decanolactone with >99% ee and 80% ee, respectively. Biotransformation of ethyl (±)-(2*E*)-4-hydroxy(4-^{2}H)dec-2-enoate yielded (4*R*)-γ-[^{2}H]decanolactone with 61% of the ^{2}H label maintained and in 90% ee indicating a stereoinversion pathway. Electron-impact mass spectrometry analysis (*Fig. 4*) of 4-hydroxydecanoic acid indicated a partial C(4) → C(2) ^{2}H shift. The formation of *erythro*-3,4-dihydroxydecanoic acid and *erythro*-3-hydroxy-γ-decanolactone from methyl (2*E*,4*S*)-4-hydroxydec-2-enoate supports a net inversion to (4*R*)-γ-decanolactone *via* 4-oxodecanoic acid. As postulated in a previous work, (2*E*,4*S*)-4-hydroxydec-2-enoic acid was shown to be a key intermediate during (4*R*)-γ-decanolactone formation *via* degradation of (3*S*,4*S*)-dihydroxy fatty acids and precursors by *Saccharomyces cerevisiae*.

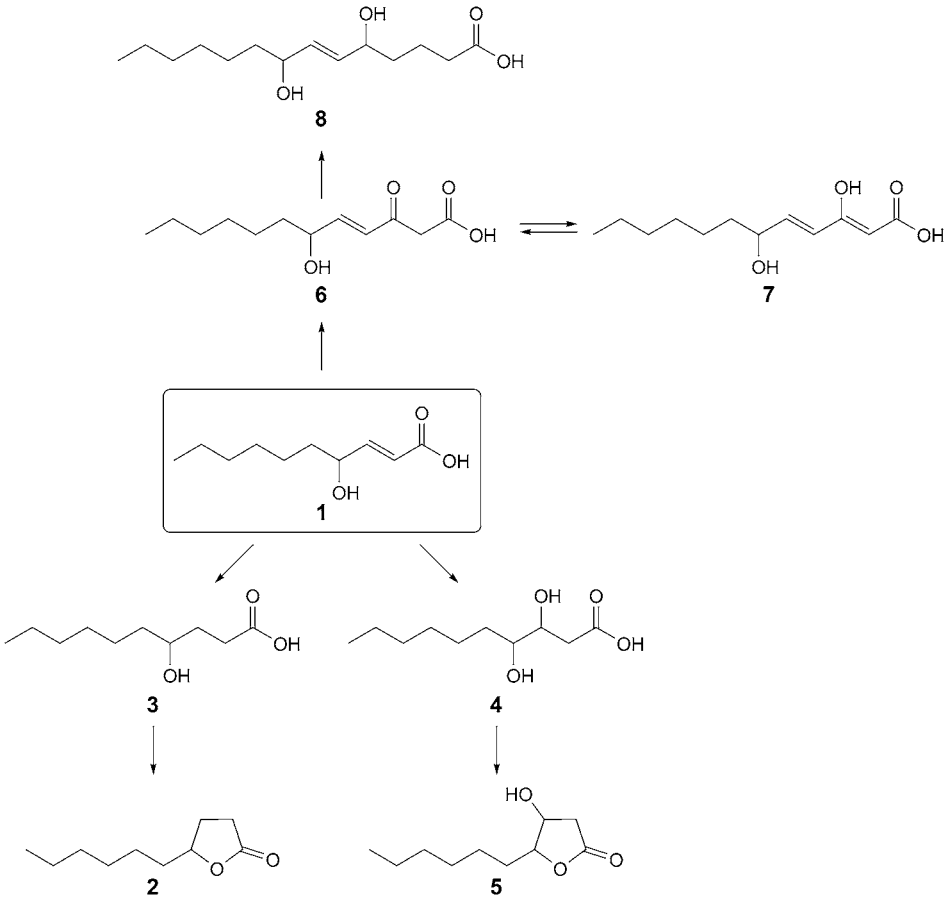
Introduction. – The β-oxidation pathway is the prevalent mechanism for fatty acid degradation in yeasts. Fatty acids carrying additional functional groups, *e.g.*, OH or epoxy residues, are metabolized with additional steps. Epoxy fatty acids are one type of precursor of the so-called 'aroma active' lactones [1–3]. Such γ- and δ-lactones represent important flavor components of different fruits [1] and act as bioactive substances bearing, for example, pheromonic character [4][5].

Epoxygenases belong to the group of monooxygenases and catalyze the formation of oxirane rings by direct addition of molecular or peroxidic oxygen to C=C bonds. Epoxy fatty acids themselves can have toxic effects on organisms, *e.g.*, coronaric and vernolic acid belongs to the group of leukotoxins [6]. In addition, they have been suggested to act as endogenous signal molecules in plant defence responses [7].

Epoxy fatty acids can also be hydrolyzed to diols by epoxide hydrolases [8][9], and are further transformed to saturated and unsaturated lactones [3]. To form these lactones, one OH group must be eliminated, and (2*E*)-4-hydroxyalk-2-enoates (allylic hydroxy fatty acids) were postulated as intermediates [3][10], *e.g.*, (2*E*)-4-hydroxydec-2-enoic acid (**1**) was suggested to lead to γ-decanolactone biosynthesis. *Gatfield et al.* investigated the formation of (4*R*)-γ-decanolactone ((4*R*)-**2**) during degradation of castor oil ((9*Z*,12*R*)-12-hydroxyoctadec-9-enoic acid glycerol ester) using *Candida lipolytica* [1]. In this instance, 3,4-dihydroxydecanoic acid (**4**) was formed, since it is not further degraded by β-oxidation. It then underwent cyclization to form 3-hydroxy-γ-

decanolactone (**5**). In addition, *Gatfield et al.* also detected dec-2-enolide and dec-3-enolide, but it remains unclear whether H_2O is eliminated from the hydroxy lactone **5** or from the linear precursor diol **4** (*Scheme 1*).

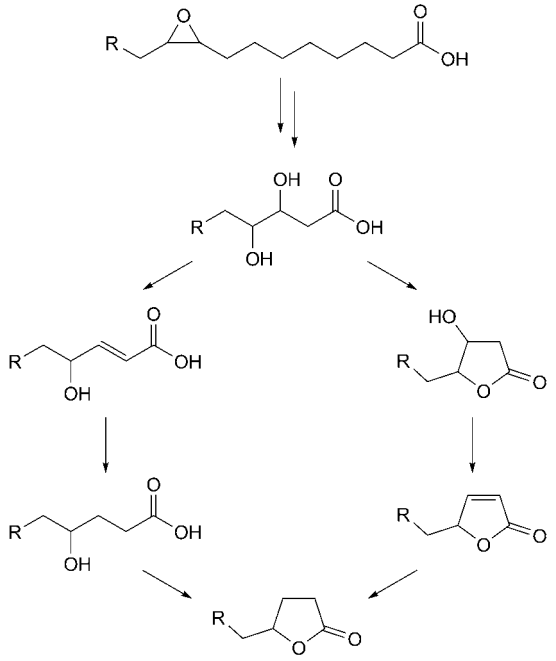
Scheme 1. *Biotransformation of* **1** *and Formation of Metabolites by* S. cerevisiae



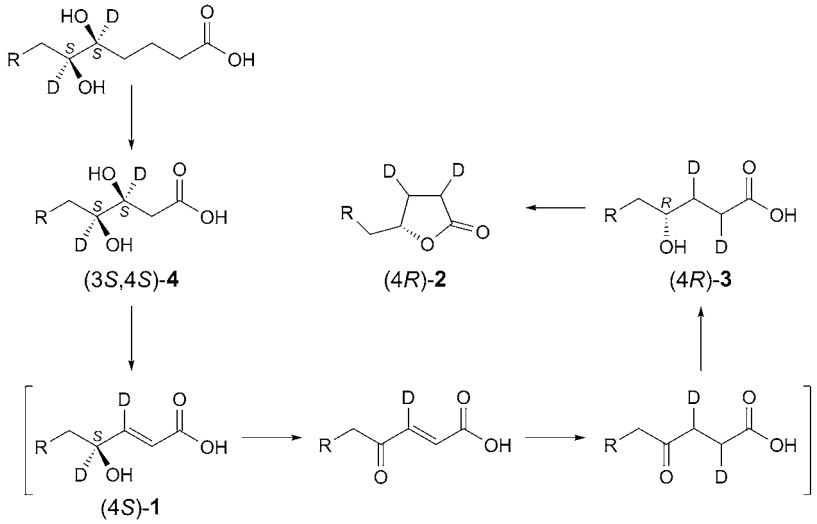
Schöttler and *Boland* studied the formation of lactones in ripening fruits [3][10]. Formation of (4*R*)-γ-undecanolactone from 9,10-epoxyheptadecanoic acid was discussed in the context of two possible pathways (*Scheme 2*). The authors favored γ-undecanolatone formation *via* the undec-2-eno-4-lactone pathway, and assumed that lactone formation *via* 4-hydroxyundec-2-enoic acid plays only a minor role [10].

Garbe and *Tressl* performed incubation experiments with *Saccharomyces cerevisiae* and showed that (4*R*)-**2** can be formed from degradation of *threo*-(5*S*,6*S*)-5,6-dihydroxydodecanoic acid [2][11] *via threo*-**4** (*Scheme 3*). However, *threo*-(5*R*,6*R*)-5,6-dihydroxydodecanoic and racemic ery*thro*-5,6-dihydroxydodecanoic acids are not metabolized into **2**. Deuterium (^{2}H) labelling clearly showed a change of the

Scheme 2. *Two Possible Pathways for γ-Undecalactone Formation from 9,10-Epoxyheptadecanoic Acid in Ripening Fruits* [3]. R = $Me(CH_2)_5$.

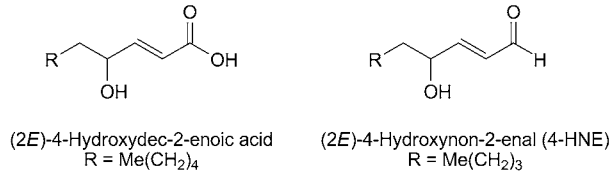


Scheme 3. *Metabolism of (5*S*,6*S*)-5,6-Dihydroxy[5,6-$^2H_2$]dodecanoic Acid in Liquid Cultures of* S. cerevisiae *and Formation of (4*R*)-[2,3-$^2H_2$]-***2*** via *Postulated Intermediate (4*S*)-[3,4-$^2H_2$]-***1*** [2]. R = $Me(CH_2)_4$.



configuration at C(4) from (*S*) to (*R*) which is accompanied by a ^2H shift from C(4) to C(2). They postulated (4*S*)-[3,4-$^2H_2$]-**1** as an intermediate in this process [2].

To date, allylic hydroxy fatty acids could neither be detected as endogenous metabolites in yeasts nor following biotransformation of epoxy fatty acids. Synthetic allylic hydroxy fatty acids were shown to exhibit cytotoxicity in human cell lines and brine shrimps [12]. They exhibit structural similarities with 4-hydroxynon-2-enal (4-HNE; *Fig. 1*) which originates from linoleic acid peroxidation. 4-HNE has been reported as a reactive cytotoxin [13], and several approaches have focussed on clarifying its mechanism of action and, consequently, reasons for 4-HNE's cytotoxicity. In this context, it has become clear that there is a link between the oxidative formation of this compound in a number of disease states, for example, diabetes mellitus [14], arteriosclerosis [15], and *Alzheimer*'s disease [16]. The protein cross-linking ability of 4-HNE was demonstrated in the literature [17], but a similar reactivity of allylic hydroxy fatty acids seems unlikely due to the significant disparity in electrophilicity between the C=O functional groups.

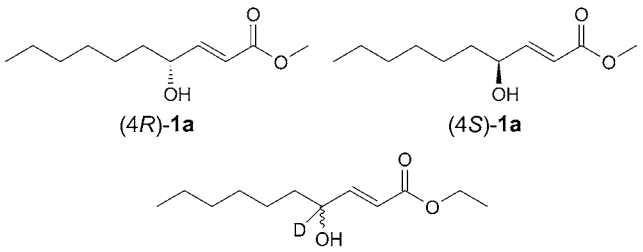


(2*E*)-4-Hydroxydec-2-enoic acid
R = $Me(CH_2)_4$

(2*E*)-4-Hydroxynon-2-enal (4-HNE)
R = $Me(CH_2)_3$

Fig. 1. *Structural similarity between substrate* **1** *and known cytotoxin 4-HNE*

To gain more insight into the metabolic fate of epoxy and dihydroxy fatty acids, (2*E*,4*R*)-4-hydroxydec-2-enoic acid, (2*E*,4*S*)-4-hydroxydec-2-enoic acid, and (±)-(2*E*)-4-hydroxy[4-^2H]dec-2-enoic acid (*Fig. 2*) were chemically synthesized and then incubated in the yeast *S. cerevisiae*. Degradation products from enantiomerically enriched and isotopically labeled substrates were identified and qualified by GC/MS analysis.



(4*R*)-**1a** (4*S*)-**1a**

(±)-[4-^2H]-**1b**

Fig. 2. *Incubation substrates*

Results. – *Chemical Synthesis.* Ethyl (±)-(2*E*)-4-Hydroxydec-2-enoate (**1b**) was synthesized in a one-pot reaction by condensation of octanal and methyl 2-(phenylsulfinyl)acetate [18], followed by transesterification. Then, (±)-[4-^2H]-**1b** was prepared

via an oxidation–deuterio reduction sequence using 4-(dimethylamino)pyridine (DMAP) · CrO_3 · HCl and subsequent $NaB[^2H]_4$ reduction. The labelling degree of (±)-[4-^{2}H]-**1b** was determined by EI-MS as > 99%.

Enantiomers of methyl (2*E*)-4-hydroxydec-2-enoate were synthesized as described in [19] through asymmetric hydroxylation of 1-(phenylsulfonyl)oct-1-ene and subsequent *Horner–Wadsworth–Emmons* reaction. 1-(Phenylsulfonyl)oct-1-ene was in turn prepared by Ru-catalyzed cross metathesis of oct-1-ene and ethenyl phenyl sulfone (for mass spectra, see *Fig. 3*).

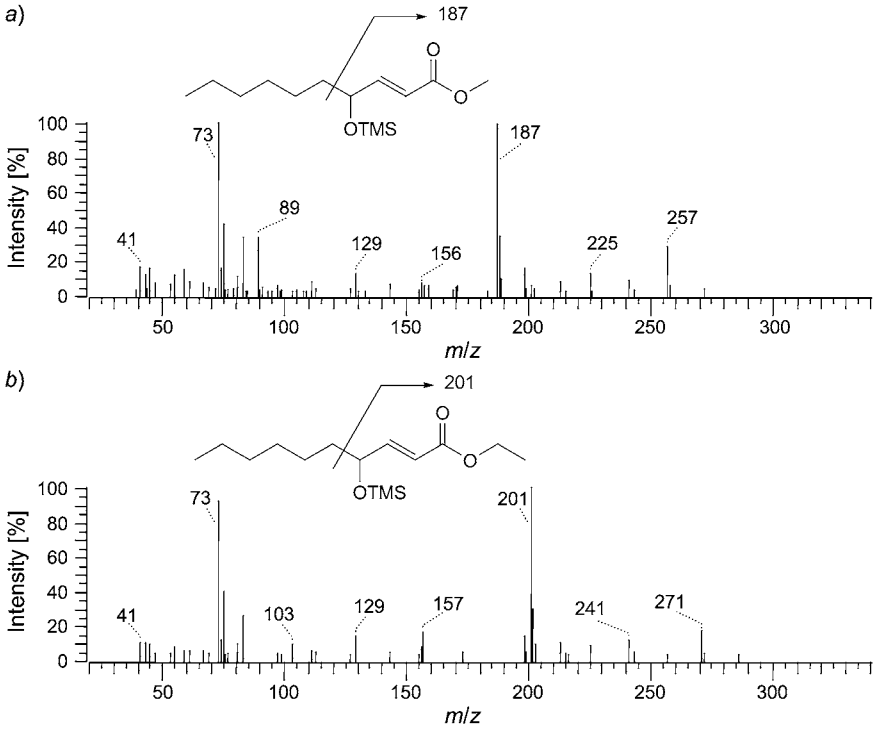


Fig. 3. *Low-resolution EI-MS of* a) *methyl (*2E*)-4-hydroxydec-2-enoate:* (synthesized, TMS ether), b) *(*2E*)-4-hydroxy[4-*^{2}*H]dec-2-enoate* (synthesized, TMS ether). TMS = Me_3Si.

Incubation Experiments. Synthetic α,β-unsaturated 4-hydroxy fatty acids were incubated as methyl (2*E*)-4-hydroxydec-2-enoate (**1a**) or as ethyl (2*E*)-4-hydroxydec-2-enoate (**1b**), because previous experiments revealed an enhanced metabolism of esters compared to free fatty acids, presumably explained by their better cell penetration.

*Metabolism of Methyl (*2E*,4*R*)-4-Hydroxydec-2-enoate* ((4*R*)-**1a**). The substrate (4*R*)-**1a** was rapidly degraded, and after 24 h of incubation only 3% of the substrate remained (*Table 1*).

(4*R*)-γ-Decanolactone (**2**) was formed and its concentration increased during incubation time. After 216 h, 12% of (4*R*)-**1a** was transformed to enantiomerically pure

Table 1. *Yields* [ppm] *of γ-Decanolactone* **2**, *Hydroxy Fatty Acid* **3**, *Dihydroxy Fatty Acid* **4**, *Hydroxy Lactone* **5**, *and Postulated Chain-Lengthened Metabolites* **6**, **7**, *and* **8** *during Biotransformation of 100 ppm (4*R*)-***1a*** in* S. cerevisiae, *Indentified and Quantified by GC/MS Analysis Using 5 ppm each of Decan-1-ol, Octano-5-lactone, and Heptadecanoic Acid as Internal Standards*

Analyte	Time [h]							
	24	48	72	96	125	168	192	216
1	3.0	1.8	2.2	0.9	0.5	0.3	0.0	1.2
2	0.1	0.8	5.6	5.5	7.3	8.8	10.4	12.0
3	0.5	8.1	9.9	4.3	3.7	3.6	3.6	4.9
erythro-**4**	0.0	0.1	0.3	0.0	0.4	0.3	0.3	0.6
erythro-**5**	0.0	0.0	1.2	1.4	1.8	3.0	3.4	4.6
6	0.0	4.7	6.6	1.1	0.0	0.8	0.7	0.3
7	21.2	26.2	32.4	35.8	20.6	23.9	21.6	13.0
8 (isomer 1)	0.0	0.6	1.0	1.0	0.0	0.5	0.2	0.0
8 (isomer 2)	0.0	1.8	2.8	0.0	0.0	0.6	0.1	0.0

(4*R*)-**2** (*Table 2*). Its direct precursor 4-hydroxydecanoic acid (**3**) was also analyzed over the entire incubation period (*Fig. 4*).

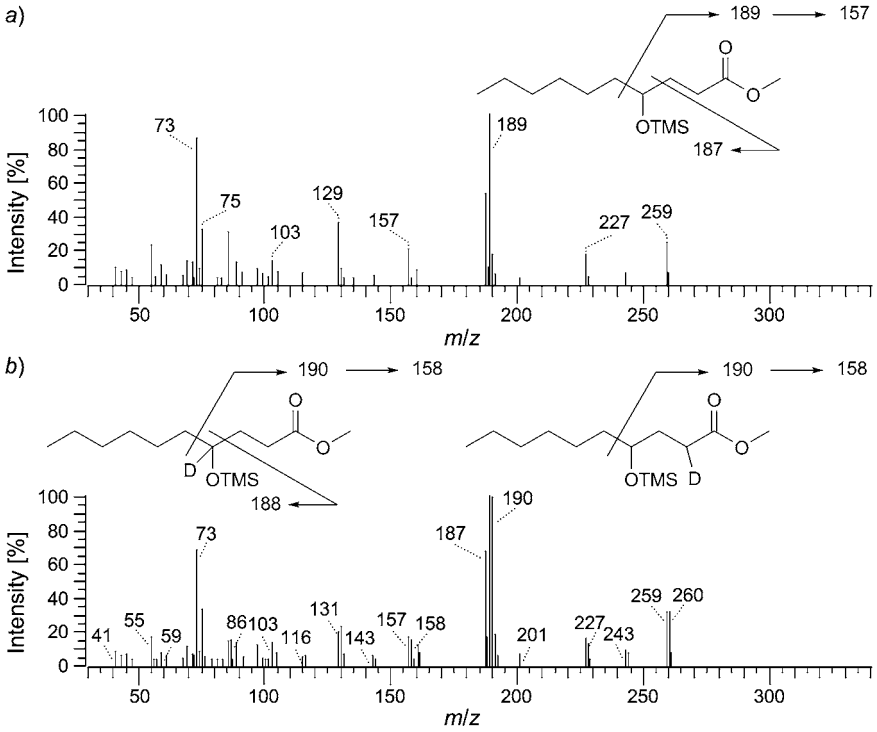


Fig. 4. *Low-resolution EI-MS of methyl 4-hydroxydecanoate* a) *unlabeled* (synthesized, TMS ether), *b*) *^{2}H-labeled: 72% total labelling, 41% C(4)-label, 31%* C(2)-*label* (isolated from liquid cultures of *S. cerevisiae* incubated with methyl (±)-(2*E*)-4-hydroxy(4-^{2}H)dec-2-enoate, TMS ether). TMS = Me_3Si.

Table 2. *Enantiomers of* **2** *during Biotransformation of* (4*R*)-**1a**, (4*S*)-**1a**, *and Racemic* [4-^{2}H]-**1b**. n.d. = Not detected.

Analyte	Biotransformation of 100 ppm of		
	(4*R*)-**1a**	(4*S*)-**1a**	(±)-[4-^{2}H]-**1b**
(4*R*)-**2**	> 99	90	95
(4*S*)-**2**	n.d.	10	5

Incubation of optically active (4*R*)-**1a** also yielded *erythro*-3,4-dihydroxydecanoic acid (*erythro*-**4**; for EI-MS-spectra see *Fig. 5*) and *erythro*-3-hydroxy-γ-decanolactone (*erythro*-**5**; for EI-MS-spectra see *Fig. 6*), thus *threo*-**4** and *threo*-**5** were not detected.

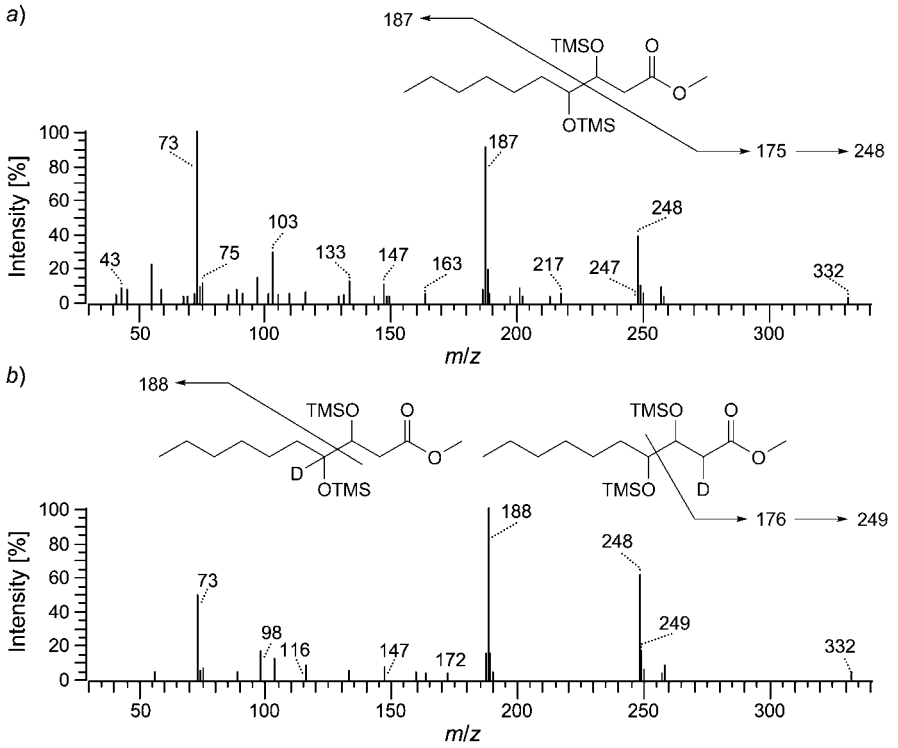


Fig. 5. *Low-resolution EI-MS of methyl 3,4-dihydroxydecanoate:* a) *unlabeled* (isolated from liquid cultures of *S. cerevisiae* incubated with methyl (2*E*,4*R*)-4-hydroxydec-2-enoate, TMS ether), b) ^{2}*H-labeled: 88% total labelling, 84%* C(4)*-label, 4%* C(2)*-label* (isolated from liquid cultures of *S. cerevisiae* incubated with methyl (±)-(2*E*)-4-hydroxy[4-^{2}H]dec-2-enoate, TMS ether). TMS = Me_3Si.

Furthermore, two notable compounds were detected by GC/MS (*Table 1*). The EI-MS (*Fig. 7*) indicated the formation of a C-chain-lengthened substrate. The first compound was postulated to be 6-hydroxy-3-oxododec-4-enoic acid (**6**). Attempts aimed at TMS ether derivatization of **6**, for GC/MS analysis, proved not straightforward due to the complication that this compound presumably exists in its enolic form

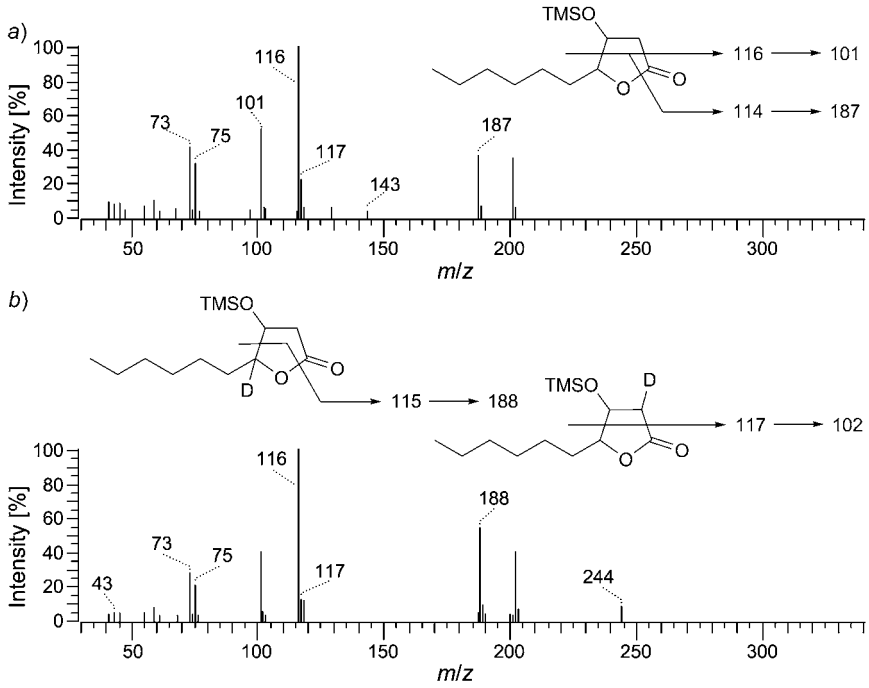


Fig. 6. *Low-resolution EI-MS of 3-hydroxy-γ-decanolactone:* a) *unlabeled* (isolated from liquid cultures of *S. cerevisiae* incubated with methyl (2*E*,4*R*)-4-hydroxydec-2-enoate, TMS ether), *b*) *^{2}H-labeled: >99% total labelling, 98%* C(4)*-label, 2%* C(2)*-label* (isolated from liquid cultures of *S. cerevisiae* incubated with methyl (±)-(2*E*)-4-hydroxy[4-^{2}H]dec-2-enoate, TMS ether). TMS = Me_3Si.

3,6-dihydroxydodeca-2,4-dienoic acid (**7**) and consequently may undergo multiple silylation processes. Nevertheless, the combined concentration of **6** and **7** reached *ca.* 40 ppm after 72 h biotransformation of (4*R*)-**1a**. The second metabolite was postulated to be 5,8-dihydroxytetradec-6-enoic acid (**8**). Two isomers of **8**, which show the same MS spectra but different GC retention times, could be separated suggesting that this compound is formed as a mixture of diastereoisomers.

*Metabolism of Methyl (2*E*,4*S*)-4-Hydroxydec-2-enoate* ((4*S*)-**1a**). Already after 24 h, the substrate (4*S*)-**1a** could not be detected (*Table 3*). The γ-lactone **2** reached a maximum concentration of 27 ppm after 216 h, which is *ca.* a 2.5-fold yield compared to the corresponding transformation of (4*R*)-**1a**. The configuration was determined as (4*R*) for **2** with 80% ee.

The maximum amount of 4-hydroxydecanoic acid (**3**), 11 ppm, was obtained after 72 h, and as time progresses the amount decreased to *ca.* 6 ppm. Based on this evidence, it seems reasonable to assume that 4-hydroxydecanoic acid **3** is the direct precursor of lactone **2**.

Compounds *threo*-**4** and *threo*-**5** were formed from (4*S*)-**1a**, both with a final concentration of 1.4 ppm. In contrast, e*rythro*-**4** and e*rythro*-**5** were only found in traces. Formation of *erythro*-**5** could only be detected after 96 h.

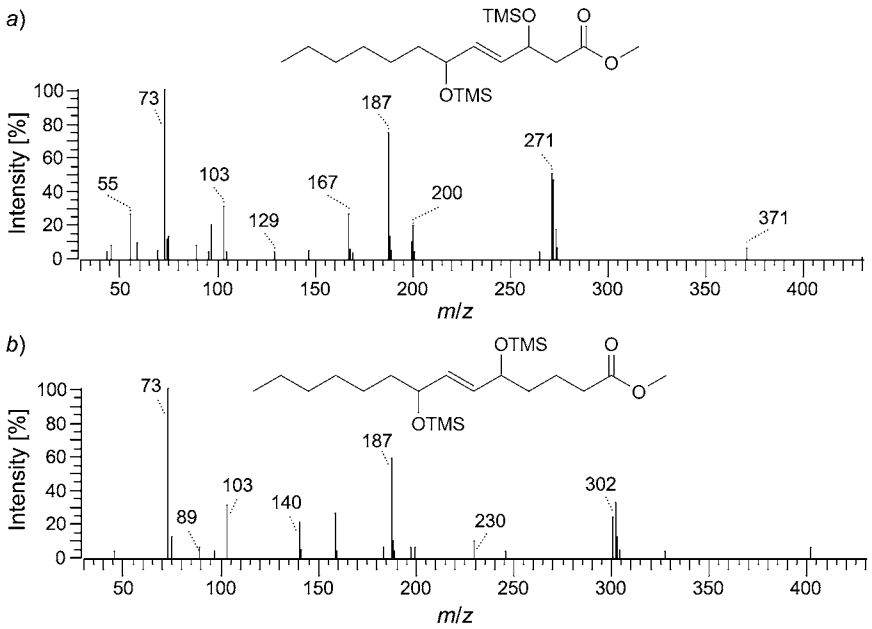


Fig. 7. *Postulated low-resolution EI-MS of* a) *methyl 3,6-dihydroxydodeca-2,4-dienoate* (isolated from liquid cultures of *S. cerevisiae* incubated with methyl (2*E*,4*R*)-4-hydroxydec-2-enoate, Me_3Si ether), *b*) *methyl 5,8-dihydroxytetradec-6-enoate* (isolated from liquid cultures of *S. cerevisiae* incubated with methyl (2*E*,4*S*)-4-hydroxydec-2-enoate, Me_3Si ether). TMS = Me_3Si.

Table 3. *Yields* [ppm] *of γ-Decanolactone* **2**, *Hydroxy Fatty Acid* **3**, *Dihydroxy Fatty Acids* **4**, *Hydroxy Lactones* **5**, *and Postulated Chain-Lengthened Metabolites* **6**, **7**, *and* **8** *during Biotransformation of 100 ppm (4*S*)-***1a*** in* S. cerevisiae, *Indentified and Quantified by GC/MS Analysis Using 5 ppm Each of Decan-1-ol, Octano-5-lactone, and Heptadecanoic Acid as Internal Standards*

Analyte	Time [h]							
	24	48	72	96	125	168	192	216
1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	0.9	3.8	14.4	16.2	17.2	19.5	19.0	27.0
3	0.0	9.8	10.5	7.5	6.6	5.5	4.7	7.2
erythro-**4**	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1
threo-**4**	0.0	0.3	0.2	0.2	0.9	0.3	1.2	1.4
erythro-**5**	0.0	0.0	0.0	0.2	0.3	0.3	0.3	0.4
threo-**5**	0.0	0.2	0.5	0.7	0.2	0.8	0.4	1.4
7	1.1	2.0	2.2	1.5	3.2	2.7	1.1	0.7
8 (isomer 1)	6.4	5.6	4.7	1.4	0.1	0.1	0.4	1.4
8 (isomer 2)	3.7	2.8	2.7	0.4	0.1	0.1	0.1	1.0

After 24 h of incubation, the postulated chain-lengthened products **7** and **8** began to accumulate. For example, **7** was detected in concentrations up to 3 ppm and both isomers of **8** together reached 11 ppm.

*Metabolism of Ethyl (±)-(2*E*)-4-hydroxy[4-^{2}H]dec-2-enoate* ((±)-[4-^{2}H]-**1b**). The substrate (±)-[4-^{2}H]-**1b** was rapidly converted and could not be detected after 15 h (*Table 4*). The γ-lactone **2** reached a concentration of 8 ppm after 140 h. The configuration was determined as (4*R*) for **2** with 90% ee. The maximum amount of 4-hydroxydecanoic acid (**3**), 17 ppm, was reached after 68 h and decreased thereafter; *threo*-**4** and *threo*-**5**, and e*rythro*-**4** and e*rythro*-**5** were formed from racemic substrate (±)-(4-^{2}H)-**1b**.

Table 4. *Yields* [ppm] *of γ-Decanolactone* **2**, *Hydroxy Fatty Acid* **3**, *Dihydroxy Fatty Acids* **4**, *Hydroxy Lactones* **5**, *and Postulated Chain-Lengthened Metabolites* **6**, **7**, *and* **8** *during Biotransformation of Acids 100 ppm (±)-[4-^{2}H]-***1b** *in S.* cerevisiae, *Indentified and Quantified by GC/MS Analysis Using 5 ppm Each of Decan-1-ol, Octano-5-lactone, and Heptadecanoic Acid as Internal Standards*

Analyte	Time [h]			
	15	68	92	140
1	0.0	0.0	0.0	0.0
2	0.0	19.4	8.0	8.3
3	0.9	16.9	3.7	1.9
erythro-**4**	0.0	2.8	0.6	0.5
threo-**4**	0.0	2.0	0.6	0.4
erythro-**5**	0.0	4.1	2.0	2.7
threo-**5**	0.0	3.7	0.9	0.6
6	0.0	0.0	1.8	0.5
7	19.7	44.4	16.6	20.7
8 (isomer 1)	7.0	13.8	0.1	0.0
8 (isomer 2)	7.3	13.3	0.4	0.0

Postulated chain lengthened products **6**, **7**, and **8** are the most abundant metabolites in the initial stage of transformation. Compound **7**, for example, was detected in concentrations up to 44 ppm, and both isomers of **8** together reached 27 ppm.

At first occurrence, γ-decanolactone (**2**) showed a ^{2}H content of 75%, which decreased slightly during the reaction time (*Table 5*). It was not possible to determine the position of ^{2}H in the lactone ring by EI-MS.

The ^{2}H content of hydroxy acid **3** also decreased from 67 to 44% during incubation time. The position of ^{2}H in **3** can be determined by EI-MS by comparison of the ion pairs 189/190 (total labelling) and 187/188 (unlabeled or labelling on C(4)). By this, partial shift of ^{2}H from C(4) to C(2) was observed. A strong ^{2}H depletion was detected at C(4) during the incubation period. In contrast, dihydroxy acid **4** and hydroxy lactone **5** were predominantly labeled at C(4) (except for *erythro*-**4**). Labelling of the chain-lengthened metabolites did not change significantly during incubation and ranged between 89–98%, whereby ^{2}H could be verified in its original position. The level of labelling matched with $[M-15]$ peak in line with total label (data not shown).

Discussion. – α,β-Unsaturated 4-hydroxy fatty acids are reactive compounds which may represent toxic species in terms of yeast metabolism. Therefore, it seems necessary for yeasts to regulate their amount. (2*E*)-4-Hydroxydec-2-enoic acid has been postulated as an intermediate within 5,6-dihydroxydodecanoic acid degradation in

Table 5. *Relative Yield* [%] *of Isotopomers of γ-Decanolactone* **2**, *Hydroxy Fatty Acid* **3**, *Dihydroxy Fatty Acids* **4**, *and Hydroxy Lactones* **5** *Isolated from Liquid Culture during Incubation of 100 ppm (±)-(4-^{2}H)-***1b** *in* S. cerevisiae, *Indentified and Quantified by GC/MS*

Analyte	Time (h)			
	68	92	116	140
Unlabeled **2**	23	28	32	34
[^{2}H]-**2**	77	72	68	66
Unlabeled **3**	33	42	28	56
[2-^{2}H]-**3**	7	9	31	11
[4-^{2}H]-**3**	60	49	41	33
Unlabeled *erythro*-**4**	50	17	19	26
erythro-[2-^{2}H]-**4**	3	8	5	7
erythro-[4-^{2}H]-**4**	47	75	76	67
Unlabeled *threo*-**4**	2	10	12	9
threo-[2-^{2}H]-**4**	0	2	4	6
threo-[4-^{2}H]-**4**	98	88	84	85
Unlabeled *erythro*-**5**	16	2	7	1
erythro-[2-^{2}H]-**5**	4	2	0	3
erythro-[4-^{2}H]-**5**	80	96	93	96
Unlabeled *threo*-**5**	9	1	0	0
threo-[2-^{2}H]-**5**	3	1	2	2
threo-[4-^{2}H]-**5**	88	98	98	98

yeast metabolism. Incubation experiments with methyl (2*E*,4*R*)-hydroxydec-2-enoate (**1a**), (4*S*)-**1a**, and ethyl (±)-(2*E*)-4-hydroxydec-2-enoate (**1b**) have been carried out to clarify the proceeding the yeast *S. cerevisiae* employs for detoxification of such substrates.

Substrate Degradation. In all experiments, the concentration of incubated substrates decreased very quickly, and only traces of **1a** or **1b** were detected during the entire incubation time. For experimental sample preparation, extracts were treated with CH_2N_2 which only derivatizes free fatty acids but does not convert esterified fatty acids, *e.g.*, phospholipids incorporated in the cell membrane, *etc*. The decrease of substrate concentration can thus be explained by partial integration into the cytosolic membrane, in addition to metabolism of the substrate. Indeed, medium-chain fatty acids often show adverse effects on the growth characteristics of yeasts, commonly explained by their integration into the cell membrane [20].

γ-Decanolactone (**2**) *Formation.* During incubation of (4*S*)-**1a**, the lactone (4*R*)-**2** is obtained with 80% ee, which is, however, significantly lower than the ee accomplished during incubation with (4*R*)-**1a** (almost 100% ee). For incubation with racemic [4-^{2}H]-**1b**, 90% ee of (4*R*)-**2** was reached. Obviously, (4*S*)-**2** originates from the substrate (4*S*)-**1a** and (4*S*)-**1b**, because (4*R*)-**1a** does not form (4*S*)-**2** at all. The overall yield of (4*S*)-**2** (5%) from racemic [4-^{2}H]-**1b** was exactly half the yield of enantiomerically pure (4*S*)-**1a** (10%). Hence, the formation *via* the initial direct reduction of the enolate double bond seems likely.

For the conversion of (4*S*)-**1** to (4*R*)-**2**, an inversion sequence, presumably *via* an oxidation/reduction step, is needed. Oxidation of (4*S*)-**1** to (2*E*)-4-oxodec-2-enoic acid by an oxidase, followed by hydrogenation now catalyzed by an enone reductase, would

lead to 4-oxodecanoic acid. The latter could be found in traces (data not shown). Subsequently, 4-oxodecanoic acid is reduced by a dehydrogenase to (4*R*)-**3**, which is further converted to (4*R*)-**2** by a lactonase.

Dihydroxy Acid and Hydroxy γ-Decanolactone Formation. β-Oxidation of fatty acids involves a reaction sequence of dehydrogenation, which leads to α,β-unsaturated acids, and subsequent hydration, which leads to β-hydroxy fatty acids. Hydratase-1 in plants, animals, and bacteria inserts this β-OH group in L-configuration. In contrast, yeasts express only hydratase-2 which catalyzes formation of the β-OH group in D-configuration. According to *CIP* priority rules, the configuration of β,γ-dihydroxy fatty acids thus biosynthesized by yeasts is (3*S*).

During incubation of substrate **1**, *threo*- and *erythro*-diols **4**, and hydroxy lactones **5** were detected. We postulate that hydratase-2 transforms (4*S*)-**1** to (3*S*,4*S*)-**4** and (3*S*,4*S*)-**5** (*threo*), and (4*R*)-**1** to (3*S*,4*R*)-**4** and (3*S*,4*R*)-**5** (*erythro*).

Previous results showed that (5*S*,6*S*)-5,6-dihydroxydodecanoic acid yielded (4*R*)-**2** with 90% ee during incubation approaches in *S. cerevisiae* [2]. In contrast to these findings, neither (5*R*,6*R*)-5,6-dihydroxydodecanoic acid [2] nor (3*R*,4*S*)- and (3*S*,4*R*)-**4** led to formation of **2** [21].

Incubation of ^{2}H-labeled (3*S*,4*S*)-**4** and its precursors (5*S*,6*S*)-dihydroxydodecanoic acid and (7*S*,8*S*)-tetradecanoic acid led to formation of (4*R*)-**2** with 90% ee. In contrast, incubation of (5*R*,6*R*)-dihydroxydodecanoic acid did not yield **2**. Stereoselectivity in this process is mediated by the elimination of the β-OH group during β-oxidation [21].

At the initial stage of biotransformation of (4*S*)-**1a**, configuration remained (4*S*), and hydratase-2 led to *threo*-**4** and *threo*-**5**. Later, also *erythro*-**4** and *erythro*-**5** were detected. An inversion of (4*S*) to (4*R*) must occur to afford *erythro*-**4**, *erythro*-**5** and (4*R*)-**2**. As mentioned above, an inversion sequence *via* oxidation/reduction of (4*S*)-**1** leading to 4-oxodecanoic acid seems likely; the following reduction leads to (4*R*)-**3**. Compound *erythro*-**4** could now be formed by normal β-oxidation (hydratase-2) and lactonized to *erythro*-**5**.

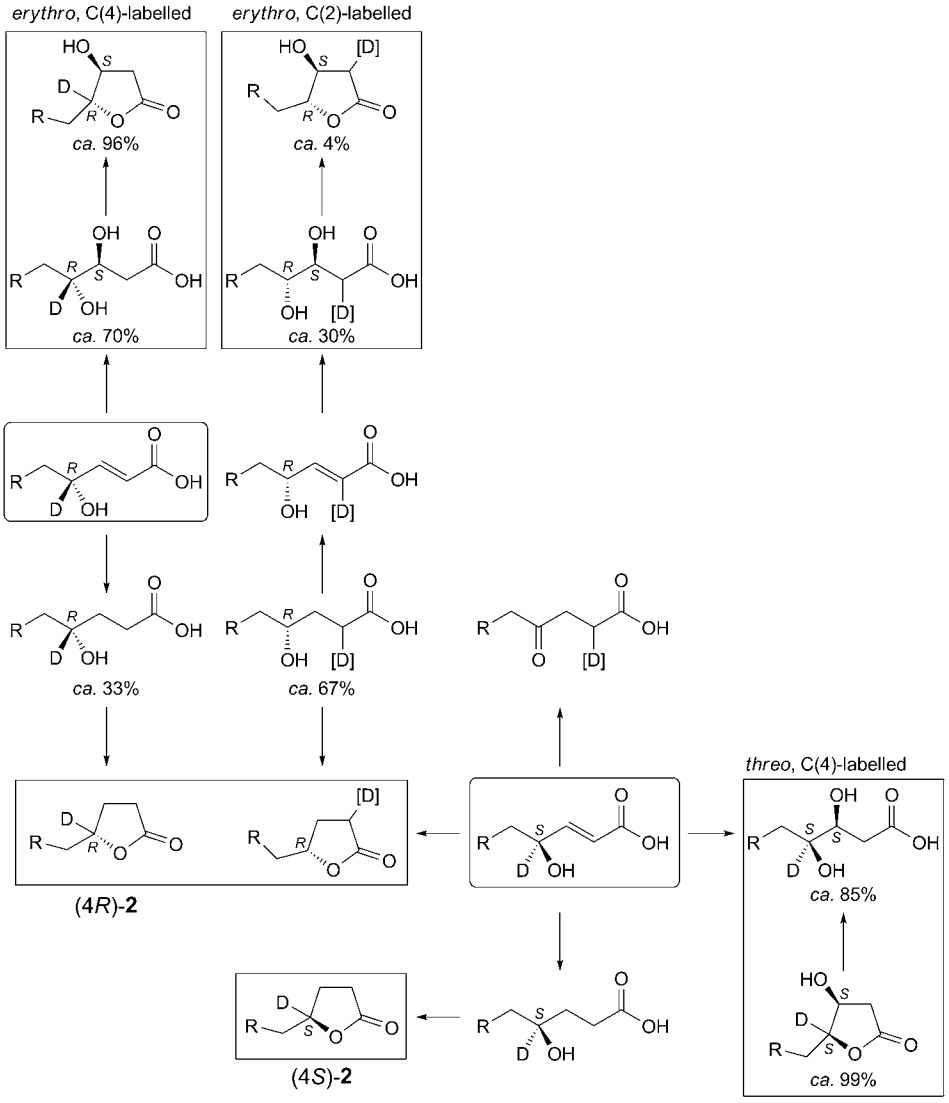
*Ethyl (±)-(2*E*)-4-Hydroxy[4-^{2}H]dec-2-enoate* ((±)-[4-^{2}H]-**1b**). Regiospecifically labeled (±)-[4-^{2}H]-**1b** was incubated in *Saccharomyces cerevisiae* to investigate the previously observed ^{2}H-shift from C(4) to C(2). This shift was observed during the metabolism of (5*S*,6*S*)-5,6-dihydroxy[5,6-$^2H_2$]dodecanoic acid [2].

Formation of *threo*-**4** and *threo*-**5** was accompanied with only a slight change of the labelling level. The position of ^{2}H in *threo*-**4** was not significantly changed. For the formation of *threo*-**4** and *threo*-**5**, it can be assumed that hardly any reactions take place which lead to an abstraction or even a shift of ^{2}H.

Compounds *erythro*-**4** and *erythro*-**5** can derive on the one hand from (4*R*)-**1b** and, on the other hand, from (4*S*)-**1b** (*Scheme 4*). For the latter, the inversion is accompanied by a decrease of ^{2}H content, and C(4) to C(2) shift. Compound *erythro*-**4** showed a slight shift of ^{2}H from C(4) to C(2), and a considerable depletion of overall ^{2}H content (*ca.* 25%), which would be expected for the proposed inversion sequence. Surprisingly, this observed ^{2}H depletion of *erythro*-**4** was not found in its successor compound *erythro*-**5**.

On the contrary, a decrease of total labelling and shifts of ^{2}H from C(4) to C(2) or C(3) had definitely been evidenced in hydroxy fatty acid **3**. A distinction between latter positions could not be achieved by MS analysis.

Scheme 4. *Metabolism of [4-^{2}H]-***1** *in Liquid Cultures of* S. cerevisiae*, and Formation of (4*R*)-***2**, *(4*S*)-***2**, **3**, erythro-**4**, threo-**4**, threo-**5**, *and* erythro-**5**. R = $Me(CH_2)_4$.



This strongly supported the previously postulated formation pathway of (4*R*)-**2** *via* **1**. The direct precursor (4*R*)-**3** to lactone (4*R*)-**2** was formed mainly by the C(4) oxidation and subsequent stereospecific oxidation pathway, a sequence accompanied with a partial ^{2}H shift and depletion (*Fig. 8*). Formation of (4*S*)-**2** proceeded *via* direct reduction of (4*S*)-**1**. Thus, a similar depletion in (4*S*)-**2** was hardly detectable.

Chain-Elongation Products. During the incubation experiments, metabolites were formed the mass spectra of which (*Fig. 7*) indicated that they were C_{12} and C_{14} hydroxy

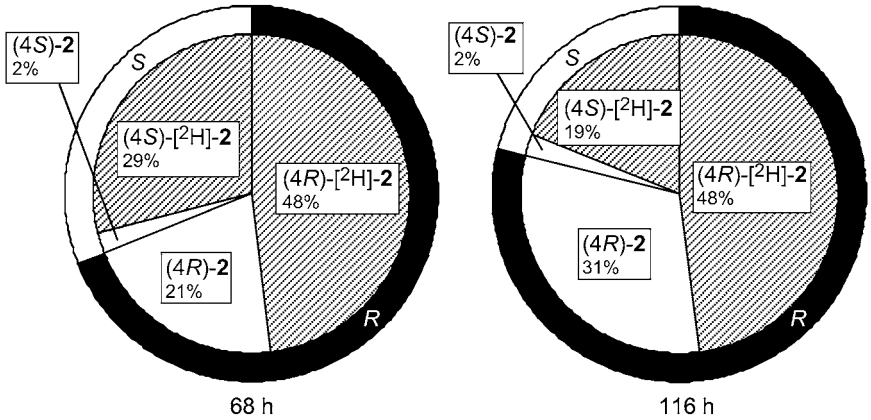
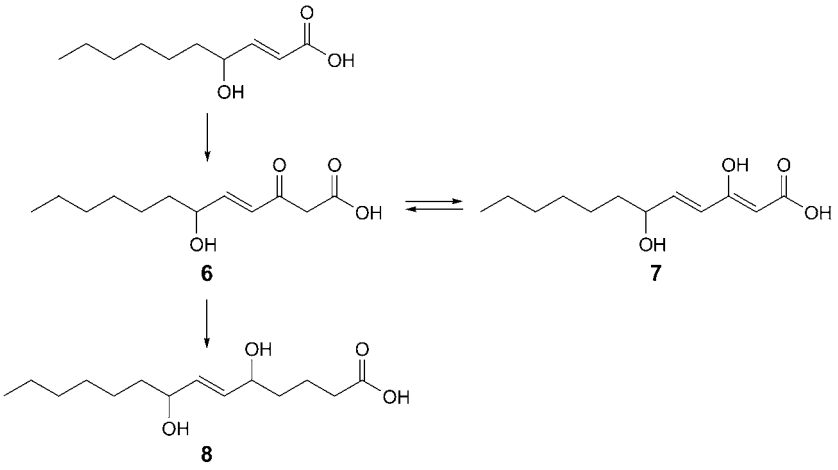


Fig. 8. *Percentage distribution of ^{2}H-labelling in γ-decanolactone* **2** *stereoisomers isolated from liquid culture after 68 and 116 h incubation of 100 ppm (±)-(4-^{2}H)-***1b** *in* S. cerevisiae*, indentified and quantified by GC/MS.*

fatty acids (*Scheme 5*). The concentration of these chain lengthened metabolites increased initially and then decreased, as medium-chain fatty acids and lactones are formed.

Scheme 5. *C-Chain Elongation Leading to Postulated Metabolites* **6**, **7**, *and* **8**



While feeding (4*R*)-**1a**, large amounts of chain lengthened C_{12}-metabolites **6** and **7** were detected, whereas C_{14}-metabolites **8** were only formed in small amounts.

On the other hand, incubation of (4*S*)-**1a** led to predominant formation of **8**. Obviously, the *S. cerevisiae* enzyme system is able to distinguish between both enantiomers during the chain-lengthening process.

As mentioned above, medium-chain fatty acids inhibit yeast growth. It seems likely that medium-chain hydroxy fatty acids are transformed to chain-lengthened metab-

olites in order to adjust their concentration. Possibly, these chain-lengthened metabolites are integrated in membranes from where they may be released gradually to form subsequent metabolites **2**–**5**. The configurational dependence in terms of the metabolic fate of these compounds is remarkable.

Experimental Part

1. *General.* ^{1}H- and ^{13}C-NMR Spectra: *AMX-500* spectrometer (*Bruker*, D-Karlsruhe,), chemical shifts δ in ppm, rel. to $CDCl_3$ (7.28 ppm) or Me_4Si (0 ppm) as internal standard, *J* in Hz, solvent, $CDCl_3$ (D: 99.5%), where applicable addition of 10% CD_3OD (D: 99.5), signal assignment by ^{1}H,^{1}H-COSY and ^{1}H,^{13}C-HETCOR. GC/MS: fused silica *DB-1* cap. column (poly(dimethylsiloxane)), 60 m × 0.32 mm i.d., 0.25 μm film (*J & W Scienitific*, Folsome, CA), temp. program starting at 70°, then 4 K/min to 280°, 135 kPa He (5.0); or *Lipodex® E* (octakis(3-*O*-butyryl-2,6-di-*O*-pentyl)-γ-cyclodextrin, 50 m × 0.25 mm i.d., 0.25 μm film (*Macherey–Nagel*, D-Düren), temp. program 10 min at 70°, then 4 K/min to 220°, 135 kPa He (5.0); *Carlo-Erba Fractovap 4160* coupled by means of a heated transfer line to a double-focusing electron-impact ionization *Varian Mat-8230* mass spectrometer, ionization energy 70 eV; *m*/*z* (intensity in %).

Sampling for GC/MS Analysis. Incubation substrates, compound for reference purposes, and metabolites were derivatized for better analysis. Polar functional groups were converted with suitable reagents to enhance volatility and thermal stability.

Methylation of Carboxyl Groups with Diazomethane. While stirring, *N*-methyl-*N*-nitroso-4-toluenesulfonamide (*Diazald®*) (5.0 g) was dissolved in Et_2O (100 ml) in a 250 ml three-necked flask. Under N_2, a 10% methanolic NaOH soln. (25 ml) was added carefully. The released gaseous CH_2N_2 was led with the aid of N_2 gas flow for 1 to 5 min into ethereal dilution of derivatizing sample. The yellow suspension was left at r.t. overnight, and, if there was no decoloration, the mixture was concentrated at 40° to an end volume of 50 μl for analysis by GC or further derivatization. If decoloration occurred, the procedure was repeated.

Silylation of OH Groups. Samples dissolved in Et_2O were concentrated under N_2 and dissolved in pyridine (50 μl). Subsequently, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; 20 μl) was added, the mixture was held for 30 min at 80° before cooling and analysis by gas GC.

α,β-Unsaturated Hydroxy Fatty Acids. Methyl (2*E*,4*R*)-4-hydroxydec-2-enoate ((4*R*)-**1a**), and methyl (2*E*,4*S*)-4-hydroxydec-2-enoate ((4*S*)-**1a**) were prepared according to a literature procedure [19].

*Ethyl (±)-(2*E*)-4-Hydroxy[4-^{2}H]dec-2-enoate* ((±)-[4-^{2}H]-**1b**). Methyl 2-(phenylsulfinyl)acetate (1.4 g, 7.3 mmol) and piperidine (0.7 g, 8.5 mmol) were dissolved in MeCN (20 ml). Octanal (1.1 g, 8.6 mmol) dissolved in MeCN (5 ml) was added slowly, and the mixture was stirred for 2.5 h at r.t. [18]. The solvent was evaporated, and the product was purified by adsorption chromatography (SiO_2; granulation 60 Å, 60 g; petroleum ether (PE)/AcOEt 9 : 1) to yielding the product methyl (±)-(2*E*)-4-hydroxydec-2-enoate (1.2 g) as a yellow oil, which was transesterificated using AcCl and EtOH (for EI-MS, see *Fig. 3*).

4-(Dimethylamino)pyridine (DMAP) · CrO_3 · HCl (5.3 g, 20,4 mmol) was dissolved in CH_2Cl_2 (15 ml), and ethyl (±)-(2*E*)-4-hydroxydec-2-enoate (760 mg, 3.5 mmol) dissolved in CH_2Cl_2 (5 ml) was gradually added, and the mixture was stirred for 20 h under exclusion of light. The mixture was diluted with Et_2O (50 ml), filtered on silica gel and evaporated. Adsorption chromatography (SiO_2, granulation 60 Å, 60 g; PE/AcOEt 9 : 1) gave ethyl (2*E*)-4-oxodec-2-enoate (170 mg) as a clear oil.

Ethyl (2*E*)-4-oxodec-2-enoate (170 mg, 0.8 mmol) was dissolved in Et_2O (20 ml). Solid NaB[$^2H_4$] (210 mg, 5.0 mmol) was added in portions, and the mixture was stirred for 22 h at 4°. D_2O (300 μl) was added, and the mixture was stirred for further 30 min at 4°. It was then acidified with sat. NH_4Cl soln. (20 ml) and 1N HCl (10 ml). The resulting aq. layer was washed with PE (10 ml), and the combined org. phases were dried (Na_2SO_4), filtered, and evaporated. Adsorption chromatography (SiO_2, granulation 60 Å, 18 g; PE/AcOEt 9 : 1) gave the labeled ethyl ester (39 mg) as a clear oil.

Yeast Strain, Culture Conditions, Sampling, and Workup. Yeast (*Saccharomyces cerevisiae* IfG-06136) was obtained from strain collection of the Institut für Gärungstechnologie, D-Berlin. The organism was stored (4°) on wort agar slants for max. 4 months. For metabolic experiments, cells were cultivated in medium (200 ml) consisting of glucose (6.0 g/l), $MgSO_4$ (3.0 g/l), $(NH_4)_2SO_4$ (2.5 g/l), KH_2PO_4 (2.5 g/l), $CaCl_2 \cdot 2\,H_2O$ (0.1 g/l), L-alanine (2.5 g/l), and yeast extract (3.0 g/l) on a horizontal shaker at 17° and 100 rpm. The pre-culture (10 ml) was inoculated into 200 ml of fresh medium, and 20 mg of methyl (2*E*,4*R*)-4-hydroxydec-2-enoate (*a*), methyl (2*E*,4*S*)-4-hydroxydec-2-enoate (*b*) and ethyl (±)-(2*E*)-4-hydroxy[4-^{2}H]dec-2-enoate (*c*), each in 200 µl of EtOH, were immediately administered to different flasks. Every 24 h up to 216 h (*a* and *b*) and 140 h (*c*), resp., 10-ml aliquots of culture broth were taken, 50 µl of internal standards (5 ppm each of decan-1-ol, octano-5-lactone, and heptadecanoic acid) were added, and then the mixture was extracted with 200 µl of Et_2O (2 × 15 ml), the extract was dried (Na_2SO_4). Prior to GC or GC/MS analysis, samples were methylated with CH_2N_2 and, if necessary, converted to derivaties (TMS-ether with BSTFA) under standard conditions.

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