Metabolism of Deuterated *threo*-Dihydroxy Fatty Acids in *Saccharomyces cerevisiae:* Enantioselective Formation and Characterization of Hydroxylactones and γ-Lactones

by **Leif-A. Garbe*** and **Roland Tressl**

Institut für Biotechnologie, Chemisch-technische Analyse, Technische Universität Berlin, Seestrasse 13, D-13353 Berlin

(phone: +49-30-45080-231; fax: +49-30-314-27544; e-mail: Leif-A.Garbe@TU-Berlin.de)

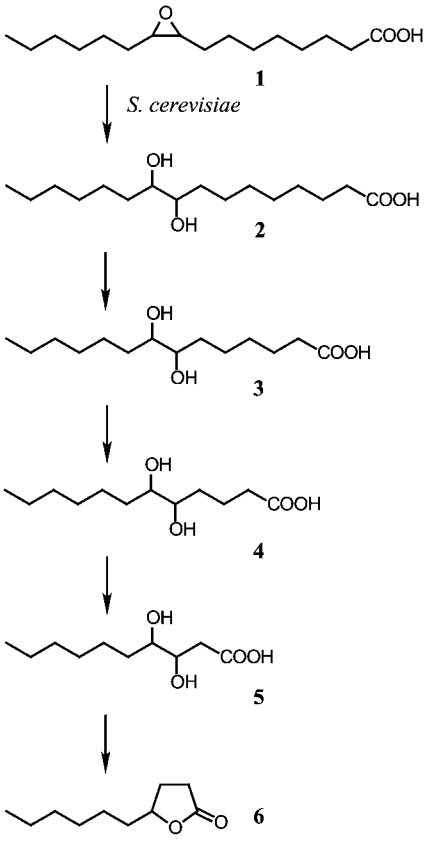
Biotransformation of (±)-*threo*-7,8-dihydroxy(7,8-$^2H_2$)tetradecanoic acids (*threo*-(7,8-$^2H_2$)-**3**) in *Saccharomyces cerevisiae* afforded 5,6-dihydroxy(5,6-$^2H_2$)dodecanoic acids (*threo*-(5,6-$^2H_2$)-**4**), which were converted to (5*S*,6*S*)-6-hydroxy(5,6-$^2H_2$)dodecano-5-lactone ((5*S*,6*S*)-(5,6-$^2H_2$)-**7**) with 80% e.e. and (5*S*,6*S*)-5-hydroxy(5,6-$^2H_2$)dodecano-6-lactone ((5*S*,6*S*)-5,6-$^2H_2$)-**8**). Further β-oxidation of *threo*-(5,6-$^2H_2$)-**4** yielded 3,4-dihydroxy(3,4-$^2H_2$)decanoic acids (*threo*-(3,4-$^2H_2$)-**5**), which were converted to (3*R*,4*R*)-3-hydroxy(3,4-$^2H_2$)decano-4-lactone ((3*R*,4*R*)-**9**) with 44% e.e. and converted to ^{2}H-labeled decano-4-lactones ((4*R*)-(3-$^2H_1$)- and (4*R*)-(2,3-$^2H_2$)-**6**) with 96% e.e. These results were confirmed by experiments in which (±)-*threo*-3,4-dihydroxy(3,4-$^2H_2$)decanoic acids (*threo*-(3,4-$^2H_2$)-**5**) were incubated with yeast. From incubations of methyl (5*S*,6*S*)- and (5*R*,6*R*)-5,6-dihydroxy(5,6-$^2H_2$)dodecanoates ((5*S*,6*S*)- and (5*R*,6*R*)-(5,6-$^2H_2$)-**4a**), the (5*S*,6*S*)-enantiomer was identified as the precursor of (4*R*)-(3-$^2H_1$)- and (2,3-$^2H_2$)-**6**). Therefore, (4*R*)-**6** is synthesized from (3*S*,4*S*)-**5** by an oxidation/keto acid reduction pathway involving hydrogen transfer from C(4) to C(2).

In an analogous experiment, methyl (9*S*,10*S*)-9,10-dihydroxyoctadecanoate ((9*S*,10*S*)-**10a**) was metabolized to (3*S*,4*S*)-3,4-dihydroxydodecanoic acid ((3*S*,4*S*)-**15**) and converted to (4*R*)-dodecano-4-lactone ((4*R*)-**18**).

Introduction. – Chiral γ-lactones, which contribute significantly to the flavors of fruits and dairy products, are bioactive substances with pheromone character. Their synthesis in microorganisms and some fruits has been investigated, and the enantiomeric distribution of γ-lactones is used to differentiate natural and artificial flavorings [1]. Biotransformation of ricinoleic acid by yeast is used on an industrial scale for the production of (4*R*)-decano-4-lactone [2]. (^{2}H)- and (^{18}O)-Labeled epoxy fatty acids were successfully transformed into optically active dodecano-4-lactones in ripening fruits, and epoxy fatty acid hydrolases were identified as key enzymes that exhibit high regio- and enantioselectivity [3–5]. In biotransformations with the lactone-producing yeast *Sporidiobolus salmonicolor*, (±)-*threo*-9,10-epoxyoctadecanoic acids were converted to (4*R*)-dodecano-4-lactone ((4*R*)-**18**) with *ca.* 90% e.e. and (±)-*erythro*-9,10-epoxyoctadecanoic acids to (4*S*)-dodecano-4-lactone ((4*S*)-**18**) with *ca.* 77% e.e. [6]. The microbial pathway for conversion of epoxy- or dihydroxy fatty acids to enantiomeric γ-lactones is largely unknown. Experiments in which (±)-vernolic acid (=(±)-(9*Z*)-12,13-epoxyoctadec-9-enoic acid) and (6*R*,7*R*)-6,7-dihydroxydodecanoic acid were incubated with *Saccharomyces cerevisiae* allowed elucidation of the metabolic course to (4*R*,5*R*)- and (4*S*,5*R*)-5-hydroxydecano-4-lactones in yeast [7]. In analogous experiments, (±)-coronaric acid (=(±)-(12*Z*)-9,10-epoxyoctadec-12-enoic acid) and (±)-*erythro*-9,10-epoxystearic acids were converted to

(4*S*,6*Z*)dodec-6-eno-4-lactone (34% e.e.) and (4*R*)-**18** (*ca.* 56% e.e.), respectively. The corresponding (±)-*threo*-9,10-dihydroxy fatty acids (**10**) were transformed into (4*R*)-**18** with >90% e.e. Therefore, the configuration of the epoxy fatty acids and their hydrolysis into dihydroxy fatty acids by epoxide hydrolases does not determine the optical purity of the lactones in yeast (*Scheme 1*).

Scheme 1. *Biotransformation of (±)-*erythro-**1** *and (±)-*threo-**2** *into Decano-4-lactones* ***6*** *in Liquid Cultures of* S. cerevisiae



Precursor (+/-)-**1**: (4*R*)-**6**, 58% e.e.

Precursor (+/-)-**2**: (4*R*)-**6**, 90% e.e.

To gain more insight into the metabolism of dihydroxy fatty acids and their degradation to γ-lactones, (±)-*threo*-7,8-dihydroxy(7,8-$^2H_2$)tetradecanoic acids ((±)-*threo*-(7,8-$^2H_2$)-**3**), methyl (5*R*,6*R*)- and methyl (5*S*,6*S*)-5,6-dihydroxy(5,6-$^2H_2$)dodecanoates ((5*R*,6*R*)- and (5*S*,6*S*)-(5,6-$^2H_2$)-**4a**), (±)-*threo*-3,4-dihydroxy(3,4-$^2H_2$)decanoic acids ((±)-*threo*-(3,4-$^2H_2$)-**5**), and methyl (9*R*,10*R*)- and methyl (9*S*,10*S*)-dihydroxyoctadecanoates ((9*R*,10*R*)- and (9*S*,10*S*)-**10a**) were synthesized and incubated in liquid cultures of *S. cerevisiae.*

Results. – 1. *Chemical Synthesis:* (±)-*threo*-(7,8-$^2H_2$)-**3** and (±)-*threo*-(3,4-$^2H_2$)-**5** were synthesized *via* catalytic deuteration (5% $PdBaSO_4$, $^2H_2$) of tetradec-6-ynoic acid and dec-3-ynoic acid followed by H_2O_2/HCOOH dihydroxylation as described in [7]. (5*R*,6*R*)- and (5*S*,6*S*)-(5,6-$^2H_2$)-**4a** were synthesized from methyl (5*E*)-(5,6-$^2H_2$)-dodec-5-enoate by *Sharpless* asymmetric dihydroxylation (*Sharpless* AD), and subsequent acid-catalyzed lactonization yielded (5*R*,6*R*)- and (5*S*,6*S*)-6-hydroxy(5,6-$^2H_2$)dodecano-5-lactones ((5*R*,6*R*)- and (5*S*,6*S*)-(5,6-$^2H_2$)-**7**). (±)-*threo*-3-Hydroxy(3,4-$^2H_2$)decano-4-lactones ((±)-*threo*-(3,4-$^2H_2$)-**9**) were synthesized by acidic lactonization of (±)-*threo*-(3,4-$^2H_2$)-**5**. Synthesis of (9*R*,10*R*)- and (9*S*,10*S*)-**10a** was achieved by *Sharpless* AD of methyl (9*E*)-octadec-9-enoate.

2. *Analysis:* The mass spectra of the substrates (±)-*threo*-(7,8-$^2H_2$)-**3**, (5*R*,6*R*)- and (5*S*,6*S*)-(5,6-$^2H_2$)-**4a**, and (±)-*threo*-(3,4-$^2H_2$)-**5** (as methyl esters, 7,8-, 5,6- and 3,4-di-*O*-(trimethylsilyl)ethers) are shown in *Figs. 1* and *2*. Typical α-cleavage reactions under EI-MS conditions are observed, and the dihydroxy fatty acid derivative showed fragmentation at the vicinal diol position. An estimate of the label content was performed by integrating the respective *m*/*z*. However, the derivative of (±)-*threo*-(3,4-$^2H_2$)-**5** provides the fragment ion *m*/*z* 187/188 ($^1H_1$,$^2H_1$), labeled *m*/*z* 188, $C_{10}H_{22}$-$^2HOSi^+$), but the corresponding α-cleavage fragment at *m*/*z* 175/176 ($C_7H_{14}{}^2HO_3Si^+$) is not prominent in the spectrum. A rearrangement of the 4-*O*-(trimethylsilyl) group (*m*/*z* 73) to the ester group results in the fragment ion *m*/*z* 248/249 ($^1H_1$/$^2H_1$), which was used to estimate the ^2H-content at C(3).

Synthesized enantiomers of *threo*-**7** can be separated on a chiral GC (*Lipodex® E*) as 6-*O*-(trifluoroacetyl)ester derivatives; (5*R*,6*R*)-**7** elutes before (5*S*,6*S*)-**7**. Enantiomers of *threo*-**9** are separated without derivatization; (3*R*,4*R*)-**9** elutes after (3*S*,4*S*)-**9**, as well as *threo*-3-hydroxydodecano-4-lactones (**16**). *Threo*-enantiomers of **9** and **16** were obtained from incubations of the (*R*,*R*)- and (*S*,*S*)-enantiomers of **4a** and **10a**, respectively. The investigation of the isotopomers of (4*R*)- and (4*S*)-**6** and (4*R*)- and (4*S*)-**18** was performed on *Lipodex® E* – GC/EI-MS. The γ-lactones showed an intense fragment ion (*m*/*z* 85, base peak) under EI-MS conditions, and the ^2H-content of the lactone ring was analyzed by integrating the MS signals *m*/*z* 85/86/87, $^1H_1$/$^2H_1$/$^2H_2$. γ-Lactones are compounds known from yeast fermentations, but 6-hydroxy-5-lactones have not previously been characterized as metabolites of the dihydroxy fatty acid metabolism. The identified 6-hydroxydodecano-5-lactones **7** showed EI-MS fragmentation comparable to that of previously characterized 5-hydroxydecano-4-lactones [7]. The ^2H-content at C(6) was analyzed by integrating the fragment ions from **7** (α-cleavage) at *m*/*z* 187/188 ($^1H_1$/$^2H_1$). As observed for 5-hydroxydecano-4-lactones, the 6-hydroxydodecano-5-lactones **7** showed rearrangement of the HO−C(6) proton and of the TMS group (*m*/*z* 73) of 6-*O*-(trimethylsilyl)ether (*m*/*z* 99 + 73/100 + 73 ($^1H_1$/$^2H_1$)) to the lactone ring moiety under EI-MS conditions. The resulting fragments *m*/*z* 172/173 ($^1H_1$/$^2H_1$) were used for quantification of ^2H-content at C(5). This effect was not observed with the 6-*O*-(trifluoroacetyl)ester derivative of **7**. These fragmentations were confirmed by the MS spectra of the corresponding 6-hydroxytetradecano-5-lactone (**13**) (*Figs. 3* and *4*), and the HO−C(6) H-transfer under EI-conditions was additionally confirmed by ^2HO−C(6) shifts in ^2H_2O (data not shown). The ^2H-content of the side chain C(6) (*m*/*z* 188/187) and of the lactone ring C(5) (*m*/*z* 173/172) was analyzed by EI-MS of the 6-*O*-(trimethylsilyl)ethers of **7**: no ^2H-depletion was

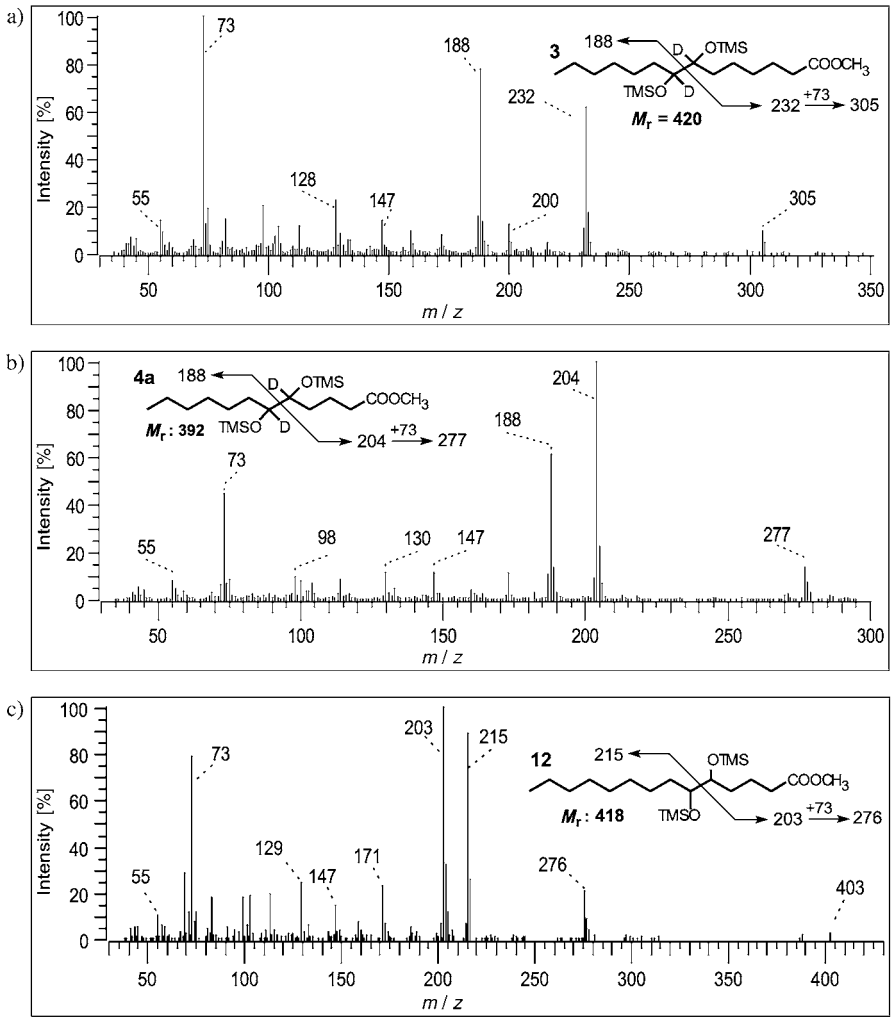


Fig. 1. *Low-resolution EI-MS of* a) *the methyl ester of (7,8-$^2H_2$)*-**3** (synthesized, Me_3Si ether), b) *the methyl ester of (5,6-$^2H_2$)*-**4a** (synthesized, Me_3Si ether), *and* c) *the methyl ester of* **12** (isolated from liquid cultures of *S. cerevisiae* incubated with (*R*,*R*)-**10a**, Me_3Si ether). TMS = Me_3Si.

observed during fermentation of (±)-*threo*-(7,8-$^2H_2$)-**3** and (5*R*,6*R*)- and (5*S*,6*S*)-(5,6-$^2H_2$)-**4a**. The MS data of the postulated 5-hydroxydodecano-6-lactones **8** are shown in *Fig. 5*. The fragmentation of the homologous ε-lactones **8** and 5-hydroxytetradecano-6-lactone (**14**) as well as of the deuterated ε-lactone ((5,6-$^2H_2$)-**8**) are in agreement with typical lactone rearrangements under EI-MS conditions. The intense fragment ions at *m*/*z* 201 in unlabeled **8** and **14** and *m*/*z* 203 in (5,6-$^2H_2$)-**8** are the result of an α-cleavage reaction with sission of the alkyl chain. The MS fragmentation of the labeled and unlabeled 3-hydroxy-4-lactones **9** and **16** are shown in *Fig. 6*. The base-peak fragment of **9** with *m*/*z* 116 (unlabeled) and an abundant peak at *m*/*z* 101 (116 – Me) is in accord

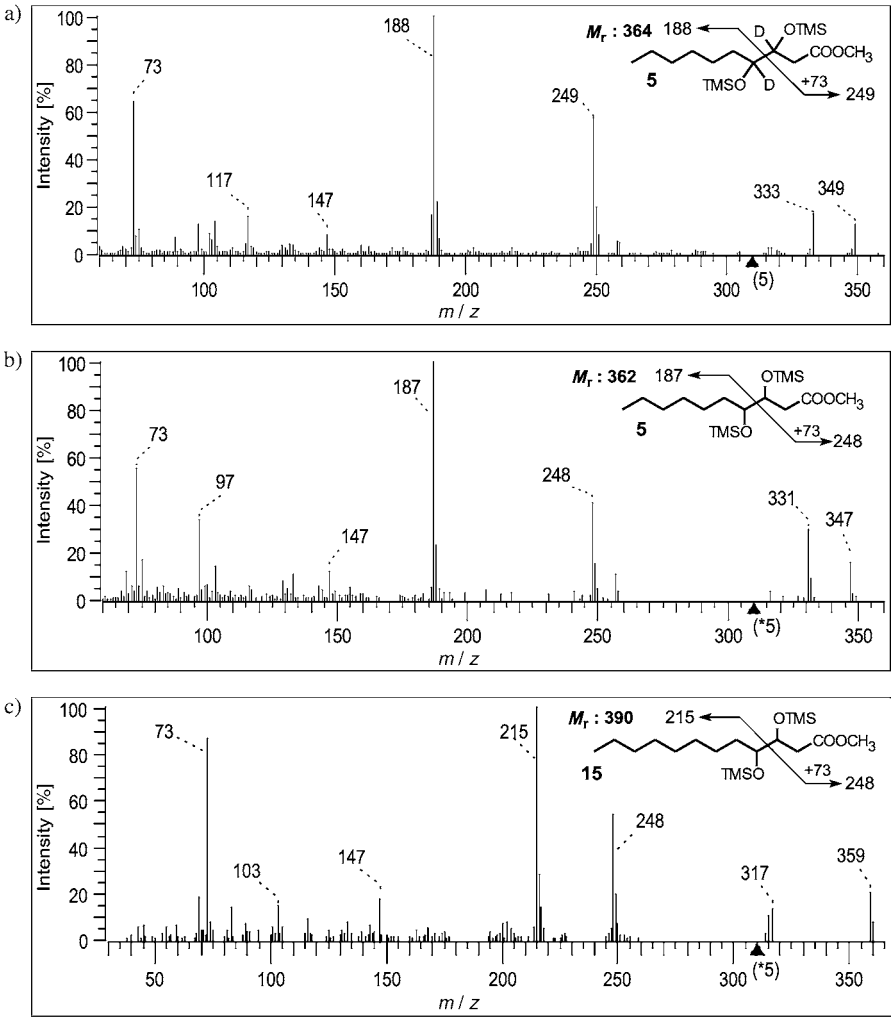


Fig. 2. *Low-resolution EI-MS of* a) *the methyl ester of (3,4-$^2H_2$)*-**5** (synthesized, Me_3Si ether), b) *the methyl ester of* **5** (synthesized, Me_3Si ether), *and* c) *the methyl ester of* **15** (isolated from liquid cultures *of S. cerevisiae* incubated with (*R*,*R*)-**10a**, Me_3Si ether). TMS = Me_3Si.

with further rearrangement of the lactone ring under EI-MS conditions. The MS spectra of 3-hydroxy-4-lactones will be discussed in a forthcoming publication.

3. *Incubation Experiments:* The racemic dihydroxy fatty acids (±)-*threo*-**3** and (±)-*threo*-**5** were incubated in yeast as free fatty acids, and the *Sharpless*-AD-derived enantiomers **4a** and **10a** were incubated as methyl esters, which were hydrolyzed readily by yeast esterases.

3.1. *Metabolism of* **3**. The results of the incubations of (±)-*threo*-(7,8-$^2H_2$)-**3** (*Fig. 1*) in liquid cultures of *S. cerevisiae* are summarized in *Tables 1* and *2*. The degradation of the substrate was nearly complete after 144 h: *threo*-(5,6-$^2H_2$)-**4** (*Fig. 1*), the

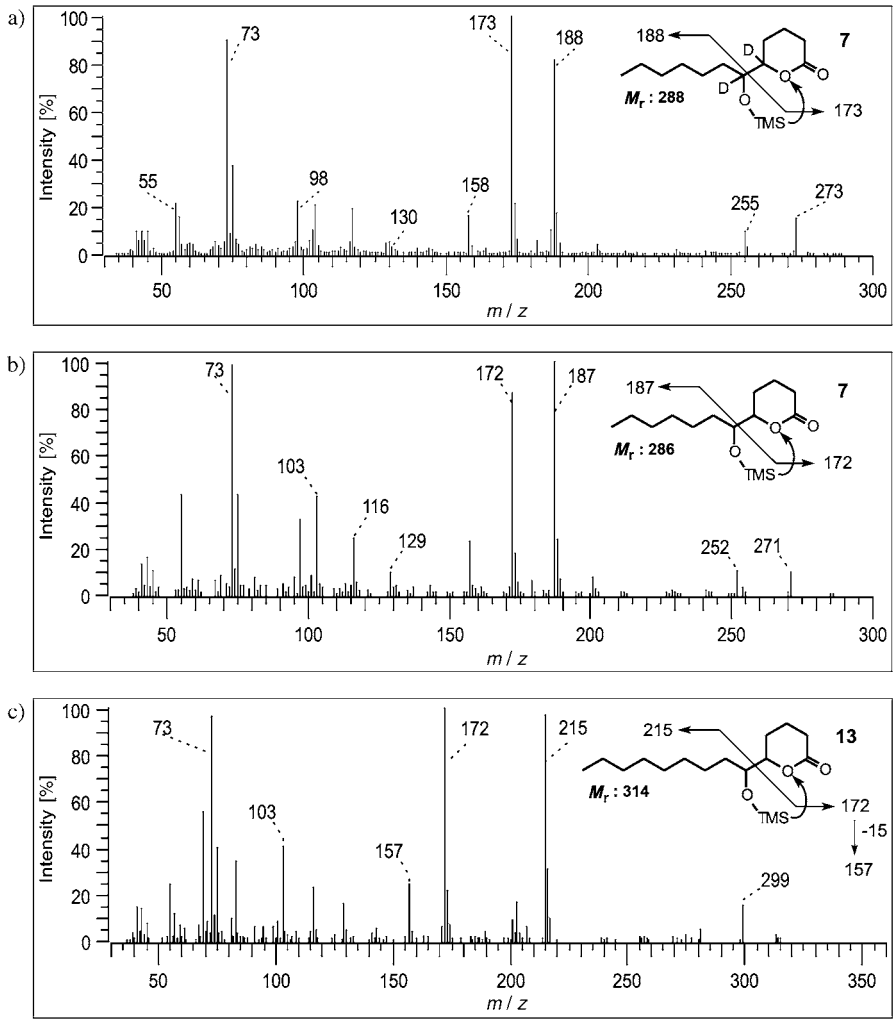


Fig. 3. *Low-resolution EI-MS of* a) *(5,6-$^2H_2$)*-**7** (synthesized, Me_3Si ether), b) **7** (synthesized, Me_3Si ether), *and* c) **13** (isolated from liquid cultures *of S. cerevisiae* incubated with (*R*,*R*)-**10a**, Me_3Si ether). TMS = Me_3Si.

corresponding labeled 6-hydroxydodecano-5-lactone **7** (*Figs. 3* and *4*), 5-hydroxydodecano-6-lactone (**8**) (*Fig. 5*), and decano-4-lactone (**6**) were analyzed as major products. The structure of **7** was confirmed by chemical synthesis whereas that of **8** is postulated on the basis of its MS fragmentation and its formation as by-product in the synthesis of **7**. Chiral discrimination (*Lipodex® E*) of the isolated hydroxy lactones indicated (5*S*,6*S*)-(5,6-$^2H_2$)-**7** with 80% e.e. and (3*R*,4*R*)-(3,4-$^2H_2$)-**9** with 44% e.e. (*Table 2*). The deuterated lactones (4*R*)-**6** accumulated to 4 ppm with 96% e.e., corresponding to 13% yield based on **3** (*Table 2*). The EI-MS method applied allowed the determination of the ^2H-content of the lactone ring but not the position of the label.

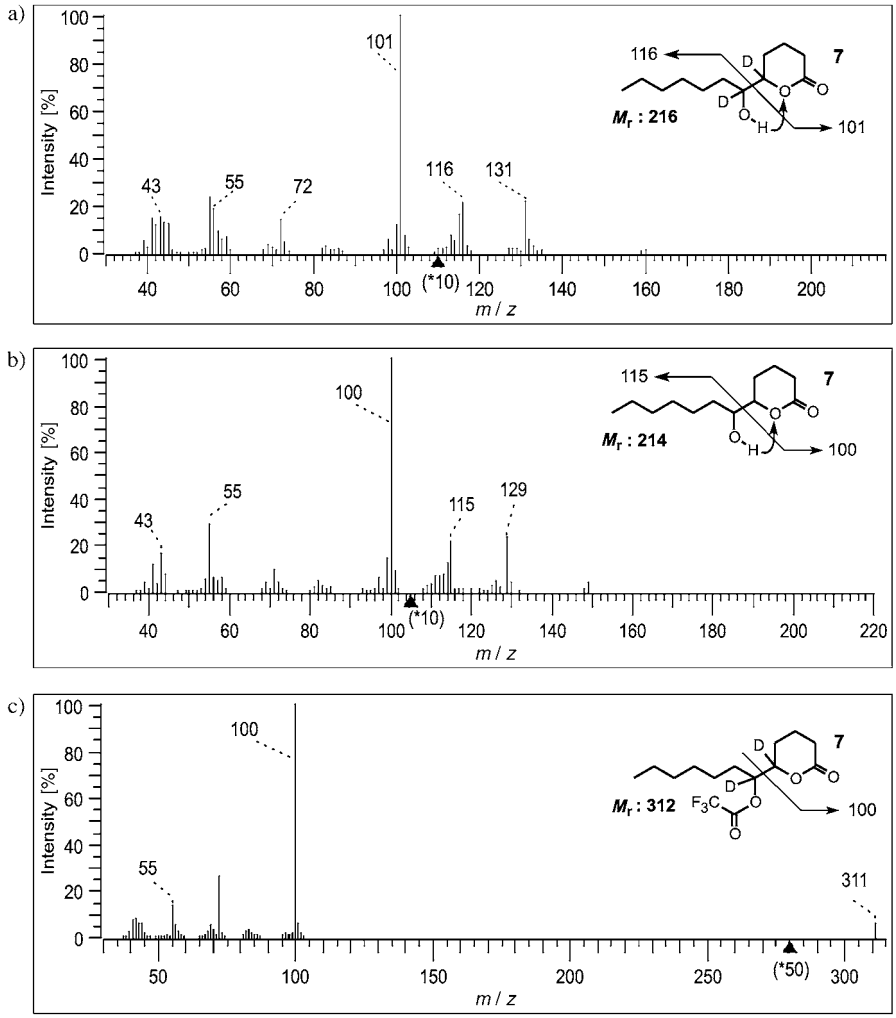


Fig. 4. *Low-resolution EI-MS of synthesized* a) *(5,6-$^2H_2$)-***7**, b) **7**, *and* c) *6-*O*-(trifluroacetyl)-(5,6-$^2H_2$)-***7**

To elucidate the labeling pattern, isolated **6** was autoclaved under basic conditions (1M NaOH, 160°, 5 h), and isotopomers of **6** were re-analyzed by EI-MS. The isotopomeric distribution ($^1H_2/^2H_1/^2H_2$) of **6** changed from 8 : 57 : 35 to 20 : 73 : 7 demonstrating that one ^2H atom exchanged due to the basic conditions and must, therefore, be localized at the acidic C(2) of **6**. Thus, the labeling pattern was assumed to be (2,3-$^2H_2$)-**6**. This is in agreement with a relative decrease in the concentration of ($^2H_2$)-**6** and increase in the concentration of ($^2H_1$)-**6** during fermentation.

3.2. *Metabolism of* **5**. To get more insight into the metabolism of dihydroxy fatty acids, the acids (±)-*threo*-(3,4-$^2H_2$)-**5** were administered as substrates (*Table 3* and *Table 4*). The main product was **6** with isotopomeric and enantiomeric ratios similar to those found during the incubation of (±)-*threo*-(7,8-$^2H_2$)-**3**, but (3*R*,4*R*)-(3,4-$^2H_2$)-**9**

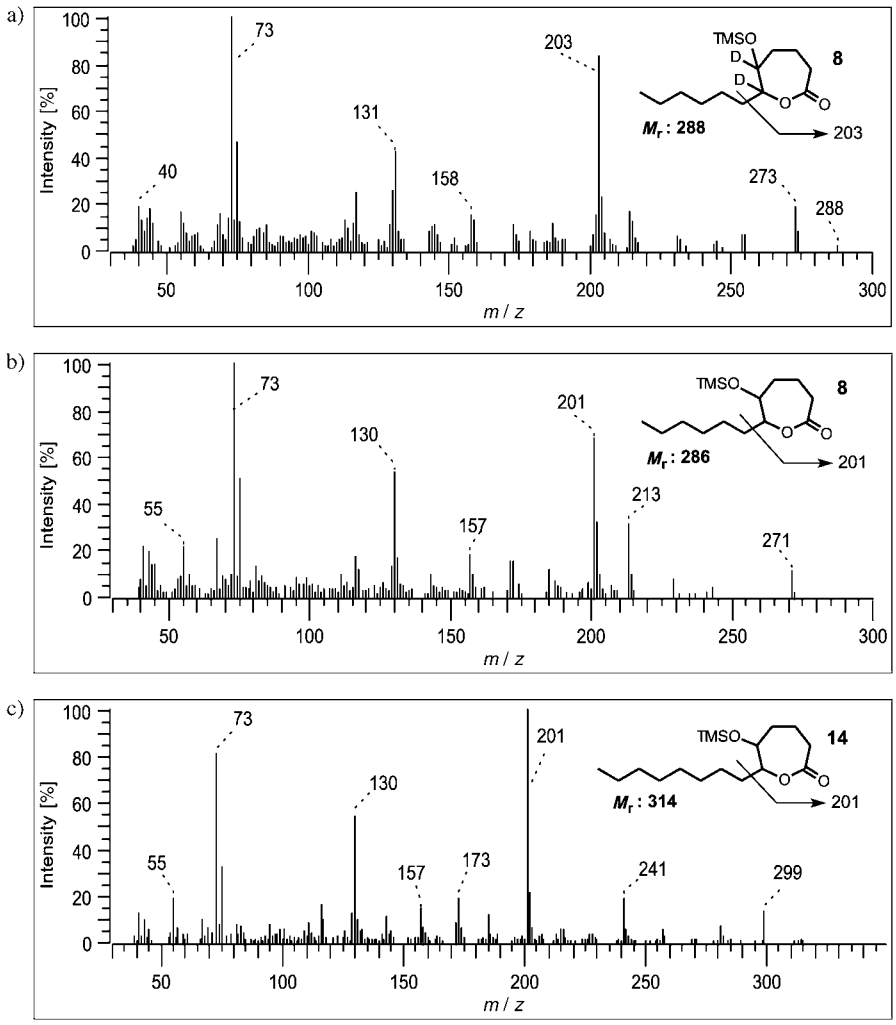


Fig. 5. *Postulated low-resolution EI-MS of* a) *(5,6-$^2H_2$)-***8**, b) **8**, *and* c) **14** (isolated from liquid cultures *of S. cerevisiae* incubated with **10a**, Me_3Si ether). TMS = Me_3Si.

was formed with lower optical purity from **5**. As a new metabolite, deuterated 4-hydroxydecanoic acid **19** was identified after 24 h in the fermentation broth. The isotopomeric distribution of the methyl ester of **19** was investigated by EI-MS (m/z = 85/86/87): unlabeled: 11%, ($^2H_1$)-labeled 33%, and ($^2H_2$)-labeled 56%. In contrast to the γ-lactones, the position of the label can be analyzed by means of fragmentation patterns in the EI-MS (*Fig. 7*). The fragment ions m/z 117, 118, and 119 result from α-cleavage and render comparable information as from the γ-lactone, but m/z 88/89/90 reveals the ^2H-content at C(2)/C(3) of **19**; the relative yield of (2,3-$^2H_2$)-**19** was found to be 51%.

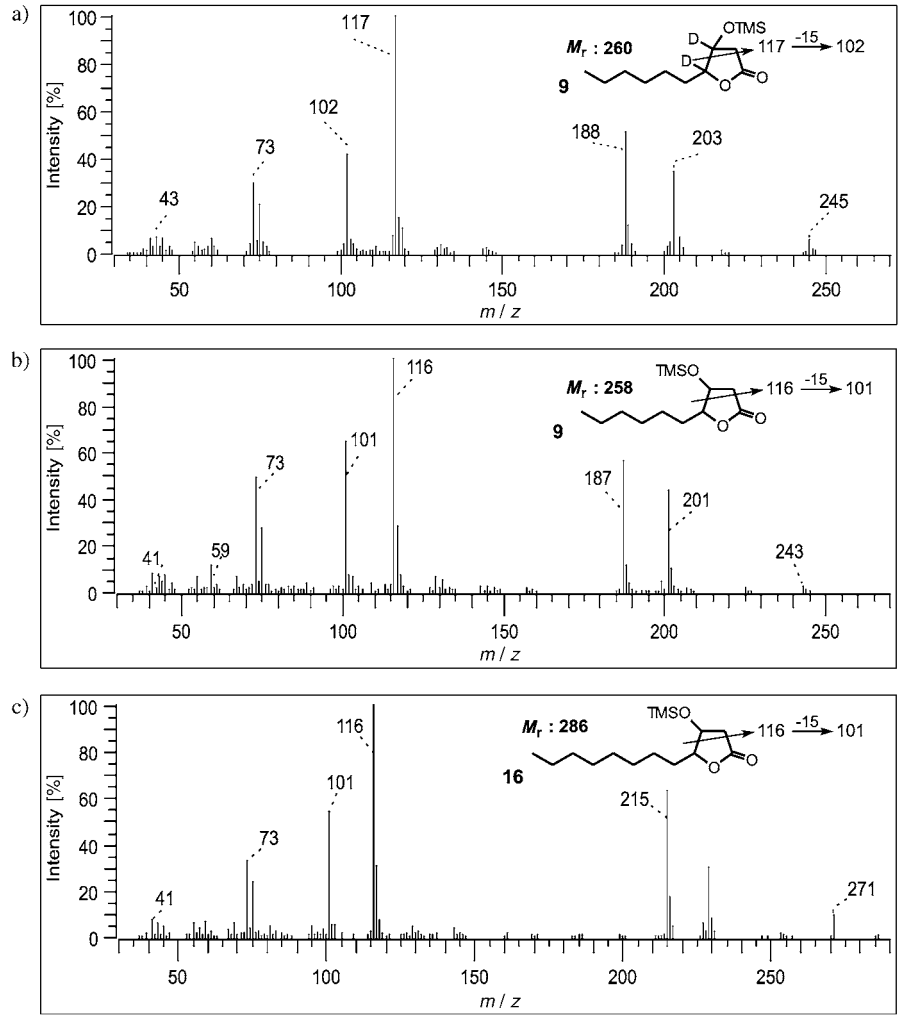


Fig. 6. *Low-resolution EI-MS of* a) *(3,4-$^2H_2$)*-**9** (synthesized, Me_3Si ether), b) **9** (synthesized, Me_3Si ether), *and* c) **16** (isolated from liquid cultures of *S. cerevisiae* incubated with **10a**, Me_3Si ether). TMS = Me_3Si.

3.3. *Metabolism of* **4a**. To identify the precursor of the (4*R*)-**6**, (5*R*,6*R*)- and (5*S*,6*S*)-**4a** were incubated with *S. cerevisiae*. The results are summarized in *Table 5*. As expected, the hydroxylactones **7**, **8**, and **9** were formed from both enantiomers in *ca.* equal amounts. However, (4*R*)-**6** was formed only from (5*S*,6*S*)-(5,6-$^2H_2$)-**4a** and possessed a distribution of ^{2}H-labeling comparable to that found after the incubation of the deuterated racemic substrates **3** and **5**. This unexpected result is in agreement with an inversion of the initial (6*S*)-configuration of **4a** and ^{2}H-transfer along the pathway of γ-lactone synthesis.

3.4. *Metabolism of* **10a**. To investigate a dependence on C-atom chain length during metabolism, the nondeuterated substrates (9*R*,10*R*)- and (9*S*,10*S*)-**10a** were adminis-

Table 1. *Yields* [ppm] *of Dihydroxy Fatty Acid* **4**, *Deuterated Hydroxylactones* **7**, **8**, *and* **9**, *and Isotopomeric Decano-4-lactones* **6** *during Biotransformation of 50 ppm (±)*-threo-*(7,8-$^2H_2$)*-**3** *by Fermentation of* S. cerevisiae

Analyte	Time [h]						
	24	48	72	144	168	264	312
(±)-*threo*-(7,8-$^2H_2$)-**3**	22.9	n.d.[a])	10.1	2.0	n.d.[a])	n.d.[a])	1.0
4	0	15.7	5.2	4.6	12.7	n.d.[a])	5.3
7	0	5.4	6.5	13.1	11.3	7.7	17.4
8	0	9.6	8.9	6.0	19.8	n.d.[a])	7.4
9	0	0	0	0	0.3	n.d.[a])	1.0
Total **6**	0	0	1.1	2.3	3.1	3.2	4.0
Unlabeled **6**[b])	–	–	5	11	12	9	10
($^2H_1$)-**6**[b])	–	–	45	43	45	52	53
($^2H_2$)-**6**[b])	–	–	50	46	43	39	37

[a]) n.d. = Not determined. [b]) Relative ratio [%] of total **6**.

Table 2. *Isotopomeric and Enantiomeric Products* **6**, **7**, *and* **9** *Isolated from Liquid Cultures of* S. cerevisiae *after Incubation of 150 ppm (±)*-threo-*(7,8-$^2H_2$)*-**3**

Analyte	Relative yield [%]		
	Total **6**	(4*R*)-**6**	(4*S*)-**6**
Total **6**	100	97	3
Unlabeled **6**	8	97	3
($^2H_1$)-**6**	57	99	1
($^2H_2$)-**6**	35	95	5
	Total **7**	(5*R*,6*R*)-**7**	(5*S*,6*S*)-**7**
(5,6-$^2H_2$)-**7**	100	10	90
	Total **9**	(3*R*,4*R*)-**9**	(3*S*,4*S*)-**9**
(3,4-$^2H_2$)-**9**	100	72	28

Table 3. *Yields* [ppm] *of Hydroxylactone* **9**, *Hydroxy Fatty Acids* **19**, *2-Hydroxyoctanoic Acid* **20**, *and Isotopomeric Decano-4-lactones* **6** *during Biotransformation of 35 ppm (±)*-threo-*(3,4-$^2H_2$)*-**5** *by Fermentation of* S. cerevisiae

Analyte	Time [h]						
	24	48	72	144	168	264	312
(±)-*threo*-(3,4-$^2H_2$)-**5**	22.1	20.7	14.5	3.4	8.2	8.0	2.2
9	9.4	n.d.[a])	n.d.[a])	n.d.[a])	1.0	n.d.[a])	n.d.[a])
19	3.3	1.1	0	0	0	0	0
20	0	0	n.d.[a])	n.d.[a])	1.1	n.d.[a])	n.d.[a])
Total **6**	1.3	3.3	4.4	n.d.[a])	8.6	10.2	10.2
Unlabeled **6**[b])	65	32	20	–	31	31	36
($^2H_1$)-**6**[b])	12	24	32	–	45	52	53
($^2H_2$)-**6**[b])	23	44	48	–	51	50	46

[a]) n.d. = Not determined. [b]) Relative ratio [%] of total **6**.

Table 4. *Isotopomeric and Enantiomeric Products* **6** *and* **9** *Isolated from Liquid Cultures of* S. cerevisiae *after Incubation of 35 ppm (±)*-threo-*(3,4-$^2H_2$)*-**5**

Analyte	Relative yield [%]		
	Total **6**	(4*R*)-**6**	(4*S*)-**6**
Total **6**	100	94	6
Unlabeled **6**	19	97	3
($^2H_1$)-**6**	32	95	5
($^2H_2$)-**6**	49	92	8
	Total **9**	(3*R*,4*R*)-**9**	(3*S*,4*S*)-**9**
(3,4-$^2H_2$)-**9**	100	55	44

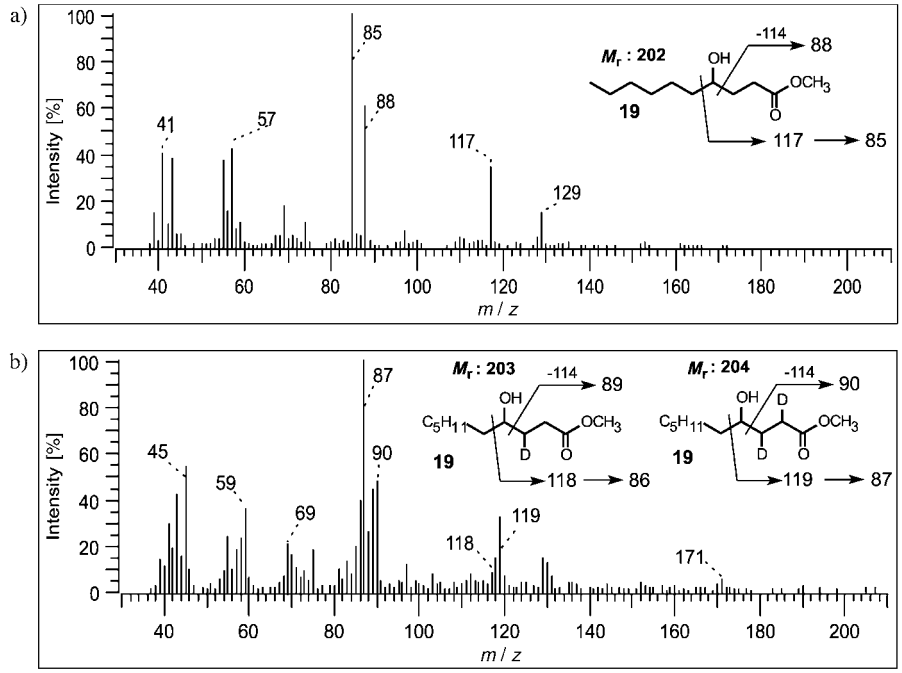


Fig. 7. *Low-resolution EI-MS of the methyl esters of* a) **19** (synthesized) *and* b) *isotopomeric* **19** (isolated from liquid cultures of *S. cerevisiae* incubated with (±)-*threo*-(3,4-$^2H_2$)-5)

tered to liquid cultures of the yeast *S. cerevisiae*. The results of the incubation experiments are summarized in *Table 6*. Both *threo*-**10a** enantiomers were comparably degraded within 96 h and the shortened dihydroxy fatty acid products (*Fig. 1* and *Fig. 2*) were analyzed by GC/MS. After 260 h fermentation, 5,6-dihydroxytetradecanoic acids **12**, the corresponding 6-hydroxytetradecano-5-lactones **13** (*Fig. 3*), and 5-hydroxytetradecano-6-lactones **14** (*Fig. 5*) were the main products in these experiments. Minor concentrations of 7,8-dihydroxyhexadecanoic acid (**11**) and 2-hydroxydecanoic acid (**17**) were detected during degradation of *threo*-**10a** enantiomers. As found for the incubation of enantiomeric **4a**, (9*R*,10*R*)-**10a** yielded only trace amounts

Table 5. *Yields* [ppm] *of Hydroxylactones* **7**, **8**, *and* **9**, *and Isotopomeric Decano-4-lactones* **6** *during Biotransformation of 150 ppm (5*R*,6*R*)- and (5*S*,6*S*)-(5,6-$^2H_2$)-***4a*** *by Fermentation of* S. cerevisiae (260 h incubation time)

Analyte	Yield [ppm]	
	(5*R*,6*R*)-**4a**	(5*S*,6*S*)-**4a**
4a	18.2	21.6
7	42.4	55.2
8	13.5	21.6
9	3.5	3.1
Total **6**	< 0.01	2.1
Unlabeled **6** (e.e. 4*R*)	–	9 (93%)
($^2H_1$)-**6** (e.e. 4*R*)	–	53 (96%)
($^2H_2$)-**6** (e.e. 4*R*)	–	38 (90%)

Table 6. *Yields* [ppm] *of Dihydroxy Fatty Acids* **11**, **12**, *and* **15**, *Hydroxylactones* **13**, **14**, *and* **16**, *and Dodecano-4-lactone* **18** *during Biotransformation of 150 ppm (9*R*,10*R*)- and (9*S*,10*S*)-***10a*** *by Fermentation of* S. cerevisiae (260 h incubation time)

Analyte	Yield [ppm]	
	(9*R*,10*R*)-**10a**	(9*S*,10*S*)-**10a**
10a	0.3	< 0.1
11	< 0.1	< 0.1
12	13.1	10.6
13	15.6	36.3
14	3.6	42.5
15	0.2	0.1
16	5.3	6.8
17	0.2	0.1
18 (e.e. 4*R*)	0.05 (n.d.[a]))	10.1 (98%)

[a]) n.d. = Not determined.

of dodecano-4-lactone **18** whereas (9*S*,10*S*)-**10a** gave rise to 10.6 ppm (4*R*)-**18**, corresponding to 12% yield based on **10a** (*Table 6*). Therefore, complete inversion of the initial (*S*)-configuration of the substrate (9*S*,10*S*)-**10a** occurs along the pathway to (4*R*)-**18**.

Discussion. – Incubation of (±)-*erythro*-9,10-epoxyhexadecanoic acids in liquid cultures of the yeast *S. cerevisiae* yielded (4*R*)-**6** with 55% e.e., whereas (±)-*threo*-9,10-dihydroxyhexadecanoic acids were converted to (4*R*)-**6** with 90% e.e. in comparable yields based on the substrates (*Scheme 1*). These results indicate that epoxy fatty acid hydrolases, which convert (±)-*erythro*-epoxy fatty acids to *threo*-dihydroxy fatty acids, do not determine the optical purity of chain-shortened hydroxylactones and γ-lactones in yeast. These results are in contrast to those found in the case of the biosyntheses of (do)decano-4-lactones in ripening fruits [3][4], where epoxide hydrolases with high regio- and enantioselectivity determine the optical purity of the γ-lactones. The peroxisomal β-oxidation in *S. cerevisiae* (by multifunctional protein (MFP) 2, 2-enoyl-CoA-hydratase-2, D-3-hydroxyacyl-CoA dehydrogenase) exhibits stereochemistry

opposite to that of the peroxisomal (microsomal) β-oxidation system of plants [8][9]. (*S*)-5-Hydroxydecanoic acids and (*S*)-3-hydroxyoctanoic acids are not metabolized by β-oxidation in *S. cerevisiae*. The (*S*)-enantiomers are degraded by α-oxidation or transformed into (*R*)-enantiomers by oxidation/keto acid reduction pathways [10]. The (±)-*threo*-**3** underwent chain shortening and lactonization to (5*S*,6*S*)-**7** with 80% e.e. and to (3*R*,4*R*)-**9** with 44% e.e., which, as far as we know, are here characterized for the first time in the β-oxidation of dihydroxy fatty acids. The result of the (5*S*,6*S*)-**4** metabolism is of particular interest, since (4*R*)-**6** was found while (5*R*,6*R*)-**4** was not converted to **6** at all. These results unequivocally prove that (4*R*)-**6** is synthesized from (3*S*,4*S*)-**5** by an oxidation/keto acid reduction pathway with H-transfer from C(4) to C(2). This pathway was confirmed with (±)-*threo*-(7,8-$^2H_2$)-**3** and (±)-*threo*-(3,4-$^2H_2$)-**5**. The identification of (2,3-$^2H_2$)-**19** in the metabolism of **5** supports a reaction sequence involving oxidation/hydrogenation of (2*E*,4*S*)-4-hydroxy(3,4-$^2H_2$)dec-2-enoic acid to 4-oxo(2,3-$^2H_2$)decanoic acid followed by reduction to (4*R*)-4-hydroxy(2,3-$^2H_2$)decanoic acid by (*R*)-dehydrogenase in yeast (*Scheme 2*), *i.e.*, transfer of $^2H_1$ from C(4) to C(2).

In the biosynthesis of lactones in ripening fruit, 3-hydroxydecano-4-lactones and (2*Z*)-dec-2-eno-4-lactones were postulated as intermediates. This pathway is obviously not operative in the conversion of (3*S*,4*S*)-3,4-dihydroxydecanoic acids to (4*R*)-decano-4-lactones in yeast, where (3*S*,4*S*)-3-hydroxydecano-4-lactone accumulated as an intermediate. The previously characterized conversion of methyl (6*R*,7*R*)-6,7-dihydroxydodecanoate to (4*R*,5*R*)- and (4*S*,5*R*)-5-hydroxydecano-4-lactones, as yeast metabolites of (±)-vernolic acids, possessed an analogous oxidation/keto acid reduction step and H-transfer from C(4) to C(2) [7].

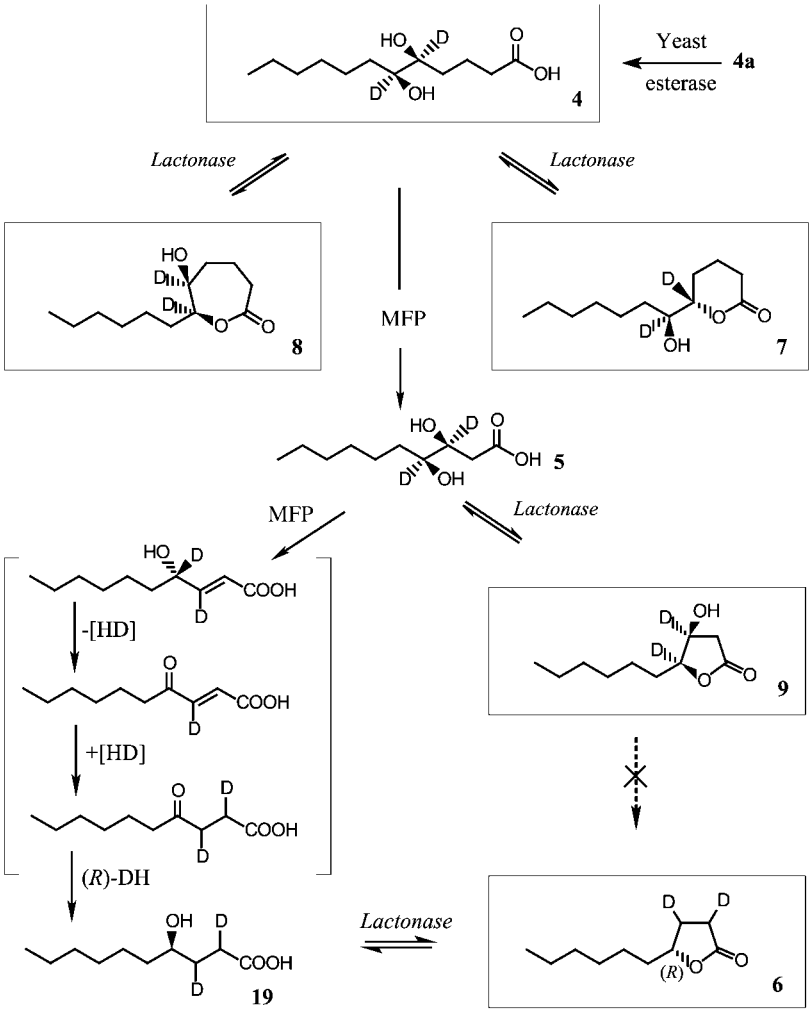
The optical purity of the γ-lactones in ripening fruits was significantly influenced by the chain length of the precursor. In yeast, the synthesis of (4*R*)-**6** from (5*S*,6*S*)-**4a** and of (4*R*)-**18** from (9*S*,10*S*)-**10a** proceeded with high optical purity and comparable yields based on the precursors (*Scheme 3*). During biotransformation of *threo*-dihydroxy fatty acids and esters, oxidation to the corresponding hydroxyoxo fatty acids but no reduction to *erythro*-dihydroxy fatty acids or *erythro*-hydroxylactones was observed. The methyl esters **4a** and **10a** were rapidly hydrolyzed by yeast and were metabolized comparably as the free fatty acids **3** and **5**. In the production of (4*R*)-**6** from ricinoleic acid with *Candida lipolytica*, *erythro*-3-hydroxydecano-4-lactone accumulated as the major metabolite as a degradation metabolite of (4*R*)-**6** [11].

Experimental Part

1. *General.* LC = Liquid chromatography. ^{1}H- and ^{13}C-NMR Spectra: *AMX-500* spectrometer (*Bruker*, Karlsruhe, Germany); chemical shifts δ in ppm rel. to $SiMe_4$ (0 ppm) as external standard, *J* in Hz, signal assignment by H,H-COSY and H,C-HETCOR. GC/MS: fused silica DB-1 capillary column (poly(dimethylsiloxane)), 60 m × 0.32 mm i.d., 0.25 μm film (*J & W Scientific*, Folsome, CA), temp. program 4 min at 80°, then 4°/min to 280°, 100 kPa He (4.0); 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 (*Machery und Nagel*, Düren, Germany), temp. program 10 min at 70°, then 4°/min to 220°) 100 kPa He (4.0); *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 %).

2. *Dihydroxy Fatty Acids.* 2.1. *(±)*-threo-*7,8-Dihydroxy(7,8-$^2H_2$)tetradecanoic Acid* ((±)-threo-*(7,8-$^2H_2$)*-**3**). As described for *threo*-6,7-dihydroxy(6,7-$^2H_2$)dodecanoic acid in [7]. 1,5-Dibromopentane (46 g, 0.2 mol) was

Scheme 2. *Metabolism of (5S,6S)-(5,6-$^2H_2$)-***4a** *in Liquid Cultures of* S. cerevisiae *and Formation of (*R*)-(2,3-$^2H_2$)-***6**



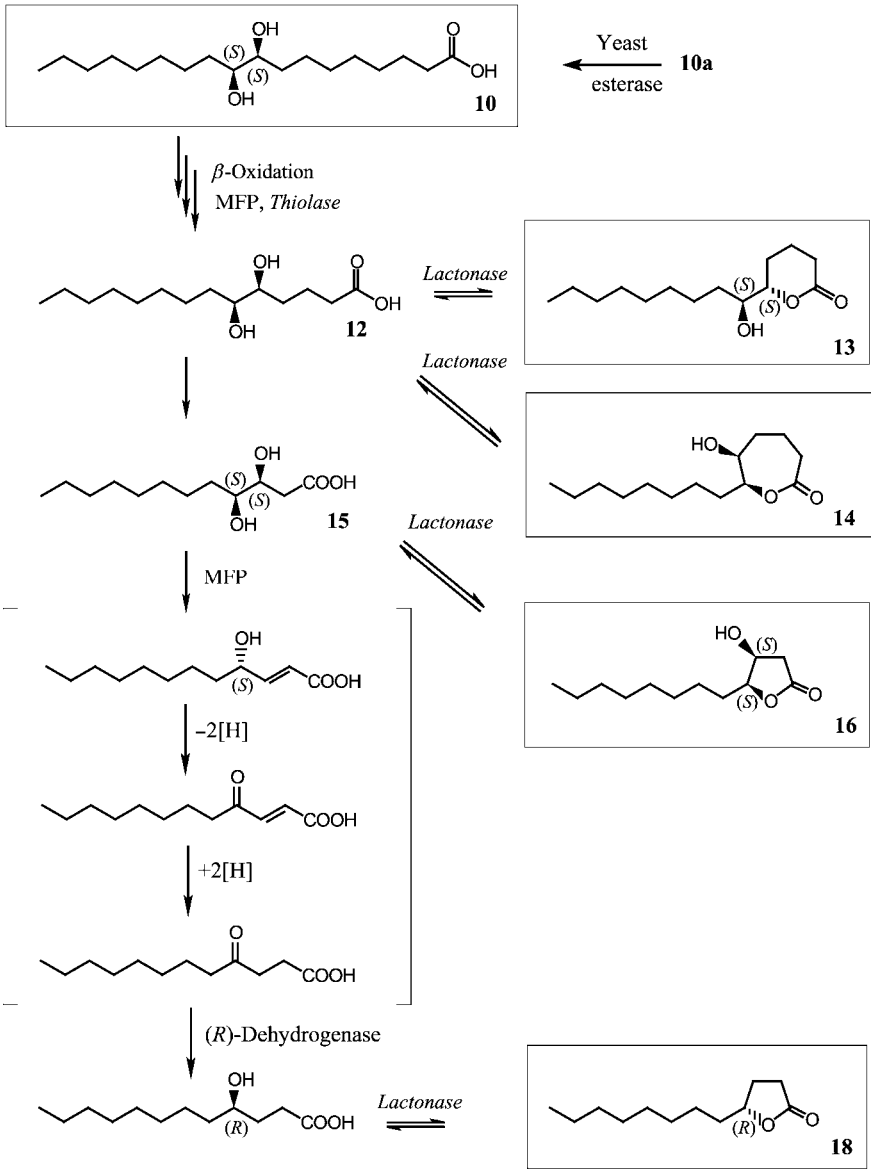
MFP = multifunctional protein

coupled with lithiated 1-octyne (1-octyne (11 g, 0.1 mol); BuLi (63 ml, 1.6M in hexane)), and the resulting 1-bromotridec-6-yne was substituted with NaCN (19.6 g, 0.4 mol) to give tetradec-7-ynenitrile (9.2 g, 45 mmol) in 45% overall yield. Saponification of the nitrile with Na_2O_2 afforded tetradec-7-ynoic acid, which was hydrogenated (5% $Pd/BaSO_4$) with $^2H_2$ gas to give (7*Z*)-(7,8-$^2H_2$)-tetradec-7-enoic acid. Dihydroxylation with H_2O_2/HCOOH yielded (±)-*threo*-(7,8-$^2H_2$)-**3** (4.5 g, 20 mmol, 45% based on tetradec-7-ynenitrile).

Tetradec-7-ynenitrile. GC/MS: 176 (5, $[M-29]^+$), 162 (10), 148 (20), 134 (25), 120 (25), 109 (10), 108 (11), 107 (15), 106 (20), 96 (10), 95 (15), 91 (15), 81 (50), 67 (85), 55 (40), 54 (50), 53 (30), 43 (25), 41 (100).

Methyl Tetradec-7-ynoate. GC/MS: 207 (5, $[M-OMe]^+$), 206 (5), 168 (20), 164 (10), 136 (30), 135 (20), 121 (20), 109 (20), 108 (20), 107 (20), 95 (40), 94 (70), 93 (40), 91 (30), 81 (50), 80 (35), 79 (70), 77 (30), 74 (20), 67 (75), 59 (50), 55 (75), 54 (40), 53 (30), 43 (70), 41 (100), 39 (40).

Scheme 3. *Metabolism of (9S,10S)*-**10a** *in Liquid Cultures of* S. cerevisiae *and Formation of (4R)*-**18**



MFP = multifunctional protein

Methyl (7Z)-(7,8-$^2H_2$)-Tetradec-7-enoate. GC/MS: 242 (10, M^+), 211 (15), 210 (20), 182 (3), 181 (3), 168 (25), 139 (15), 126 (20), 125 (21), 112 (22), 111 (30), 110 (20), 99 (30), 98 (55), 97 (60), 96 (40), 87 (50), 85 (40), 84 (60), 83 (50), 82 (30), 74 (100), 70 (40), 69 (50), 68 (40), 59 (40), 57 (40), 56 (80), 55 (90), 43 (90), 42 (50), 41 (70).

Data of (±)-threo-**3**. ^{1}H-NMR: 3.21 (*m*, 0.14 H, H–C(7), H–C(8), *i.e.*, 93% labeling); 2.21 (*t*, *J* = 7, CH_2COOH); 0.89–1.51 (*m*, 18 H, CH_2), 0.81 (*t*, *J* = 7 Me).

2.2. *Asymmetric Dihydroxylation* (AD): *Formation of Methyl* threo-*9,10-Dihydroxyoctadecanoate*s (threo-**10a**) *and Methyl* threo-*5,6-dihydroxy(5,6-$^2H_2$)dodecanoates* (threo-*(5,6-$^2H_2$)*-**4a**). AD was performed by the '*Sharpless* AD' method essentially as described in [7]. Methyl (*E*)-alkenoates yielded (*S*,*S*)-dihydroxy fatty acids methyl esters with 'AD-Mix α' and (*R*,*R*)-dihydroxy fatty acid methyl esters with 'AD-Mix β'.

*Methyl (5*E*)-(5,6-$^2H_2$)Dodec-5-enoate. 1-Bromoundec-4-yne* substituted with NaCN gave the nitrile, which was saponified (see *Exper. Part 2.1.*). *Dodec-5-ynoic acid* was reduced to *(5*E*)-(1,1,5,6-$^2H_4$)dodec-5-enol* with $LiAl(^2H)_4$ in diglyme at 160° for 72 h. Oxidation of the alcohol with pyridinium chlorochromate (PCC and $HClO_2$) and methylation with HCl/MeOH yielded *methyl (5*E*)-(5,6-$^2H_2$)dodec-5-enoate*, similar to products reported in [7].

1-Bromoundec-4-yne. GC/MS: 151 (3, $[M-Br]^+$), 134 (2), 121 (2), 109 (25), 95 (100), 93 (15), 91 (15), 82 (10), 81 (60), 79 (30), 77 (25), 69 (15), 68 (15), 67 (90), 65 (15), 55 (45), 54 (55), 53 (35), 52 (20), 51 (15), 43 (15), 42 (10), 41 (100), 40 (10), 39 (60).

*Methyl (5*E*)-(5,6-$^2H_2$)Dodec-5-enoate*. 214 (5, M^+), 183 (5), 182 (15), 165 (10), 164 (10), 140 (25), 139 (15), 130 (10), 112 (15), 111 (20), 101 (10), 100 (15), 99 (20), 98 (40), 97 (40), 87 (20), 85 (30), 84 (35), 83 (40), 82 (30), 74 (95), 71 (20), 70 (30), 69 (50), 59 (40), 56 (60), 55 (60), 43 (100), 42 (50), 41 (85).

*(5*E*)-(5,6-$^2H_2$)Dodec-5-enoic Acid*. ^{1}H-NMR: 2.24 (t, $J=7$, CH_2); 1.61 (*quint.*, $J=7$, CH_2); 1.94 (t, $J=6.5$, CH_2); 5.5–5.7 (2m, 0.068 H, H–C(5), H–C(6), *i.e.*, 96.6% labeling); 1.89 (t, $J=6.5$, CH_2); 1.1–1.4 (m, 4 CH_2); 0.81 (t, $J=7$, Me).

*(5*Z*)-Dodec-5-enoic Acid.* ^{1}H-NMR ($CDCl_3$): 5.39 (*dt*, $J=10$, 7, CH); 5.29 (*dt*, $J=10$, 7, CH); 2.35 (t, $J=7$, CH_2); 2.07 (q, $J=7$, CH_2); 1.94 (q, $J=7$, CH_2); 1.68 (*quint.*, $J=7$, CH_2); 1.2–1.4 (m, 4 CH_2); 0.83 (t, $J=7$, Me). ^{13}C-NMR: 180.21 (C(1)); 131.36 (C(5)); 128.08 (C(6)); 33.39; 31.73 (C(7)); 29.62; 28.96; 27.21; 26.37; 24.54; 22.63; 14.10 (C(12)).

*Methyl (9*S*,10*S*)-9,10-Dihydroxyoctadecanoate* ((*9*S*,10*S*)-**10a**). ^{1}H-NMR ($CDCl_3$): 3.65 (s, OMe); 3.37 (m, 2 CH); 2.28 (t, $J=6.5$, CH_2); 1.59 (*quint.*, $J=6.5$, CH_2); 1.50–1.19 (m, 12, CH_2); 0.85 (t, $J=7$, Me). ^{13}C-NMR: 174.31 (C(1)); 51.41 (OMe); 74.43, 74.49 (C(9), C(10)); 34.03; 33.61; 33.55; 31.83; 29.66; 29.52; 29.39; 29.23; 29.11; 29.00; 25.64; 25.53; 24.85; 22.62; 14.10 (C(18)).

The enantiomer (9*R*,10*R*)-**10a** showed the same NMR spectra.

2.3. *(±)-3,4-Dihydroxy(3,4-$^2H_2$)decanoic Acid* (*(3,4-$^2H_2$)*-**5**). *2-(But-3-ynyloxy)tetrahydropyran*. At 20°, but-3-yn-1-ol (7 g, 100 mmol) was mixed with 3,4-dihydro-2*H*-pyran (16.8 g, 200 mmol) and toluene-4-sulfonic acid monohydrate (5 g) in 50 ml Et_2O and stirred for 24 h. The mixture was extracted with sat. $NaHCO_3$ (50 ml), dried (Na_2SO_4), and evaporated: 13.1 g (85%) *2-(but-3-ynyloxy)tetrahydro-2*H*-pyran*. GC/MS: 153 (3, $[M-1]^+$), 126 (5), 125 (5), 115 (10), 101 (8), 99 (15), 96 (13), 95 (8), 85 (100), 83 (10), 79 (15), 67 (25), 57 (25), 56 (28), 55 (30), 54 (15), 53 (55, $[M-OTHP]$), 52 (15), 43 (25), 41 (57), 39 (25).

2-(Dec-3-ynyloxy)tetrahydropyran. At 0° under N_2, BuLi (24 ml, 2.5M in hexane, 60 mmol) was added dropwise to the crude 2-(but-3-ynyloxy)tetrahydropyran (8 g, 52 mmol) in THF (50 ml), stirred for 2 h and 1-bromohexane (16.5 g, 100 mmol) in *N*,*N*-dimethylpropylurea, (DMPU = 3,4,5,6-tetrahydro-1,3-dimethylpyrimidine-2(1*H*)-one; 60 ml) were added, stirred for 24 h and heated for 2 h to reflux. The mixture was hydrolyzed with ice water (200 ml), extracted twice with 50 ml petrolium ether, and the combined org. phases were washed three times with brine (50 ml), dried (Na_2SO_4) and evaporated: 14 g 2-(dec-3-ynyloxy)tetrahydropyran (contains 40% 1-bromohexane). GC/MS: 153 (3, $[M-85]^+$), 115 (10), 101 (8), 95 (9), 85 (100), 81 (15), 79 (10), 69 (8), 68 (7), 67 (25), 57 (15), 56 (8), 55 (20), 43 (22), 41 (30), 39 (18).

Dec-3-yn-1-ol. At 20°, the crude 2-(dec-3-ynyloxy)tetrahydropyran (14 g) and toluene-4-sulfonic acid (3 g) were dissolved in MeOH (300 ml) and stirred for 24 h. The mixture was evaporated, diluted with H_2O (300 ml) and extracted three times with 50 ml Et_2O. The combined org. phases were washed with sat. $NaHCO_3$ (100 ml) and brine (50 ml), dried (Na_2SO_4), and evaporated. The crude alcohol was purified by *Kugelrohr* distillation (Kp 100°, 3 mm Hg): 6.1 g (39 mmol, 76%) dec-3-yn-1-ol. GC/MS: 123 (5, $[M-CH_2OH]^+$), 121 (8), 109 (9), 108 (8), 107 (19), 97 (20), 95 (15), 93 (15), 91 (12), 84 (28), 83 (13), 81 (48), 80 (10), 79 (33), 77 (20), 69 (45), 68 (35), 67 (55), 66 (15), 65 (17), 56 (20), 55 (75), 54 (65), 53 (35), 52 (20), 51 (18), 45 (20), 44 (15), 43 (60), 42 (25), 41 (100), 40 (12), 39 (55), 31 (57).

*(3*Z*)-(3,4-$^2H_2$)Dec-3-enoic acid*. At 20°, CrO_3 (5.5 g, 55 mmol) and conc. H_2SO_4 (4.8 ml) were dissolved in H_2O (18.6 ml), and dec-3-yn-1-ol (6.1 g, 39 mmol) in acetone (110 ml) were added during 1 h while the temp. was held at 20° and stirred for 15 h. The mixture was evaporated, diluted with H_2O (50 ml) and extracted three times with Et_2O. The combined ether phases were washed three times with brine (each 40 ml), dried (Na_2SO_4) and the crude acid was purified by LC (36 g silica gel, 300 ml PE/EA/HOAc 18 : 2 : 0.2): 1.2 g (18%) pure dec-3-ynoic acid.

(±)-threo-*3,4-Dihydroxy(3,4-$^2H_2$)decanoic Acid* (*(±)*-threo-*(3,4-$^2H_2$)*-**5**). At 0°, 5% Pd/$BaSO_4$ (30 mg) was suspended in 40 ml MeOH, quinoline (0.35 ml) was added, and the mixture was evacuated and aerated with $^2H_2$ gas (*Messer-Griesheim*, Germany, 2.7). Addition of dec-3-ynoic acid in MeOH (1 ml) (0.68 g, 4 mmol) started the consumption of the theoretical volume of 90 ml $^2H_2$. The catalyst was filtered off, the filtrate was diluted with Et_2O (50 ml) and H_2O (50 ml) and acidified with 2M HCl. The org. phase was washed (brine), dried (Na_2SO_4), and evaporated, and the crude acid (0.62 g, 92%) was directly subjected to dihydroxylation with H_2O_2 in conc. formic acid as described in [7]. LC of the crude deuterated dihydroxy acid yielded only 15 mg (±)-*threo*-(3,4-$^2H_2$)-**5** (*Fig. 2*).

3. Hydroxylactones: 3,4- and 5,6-Dihydroxy fatty acids or their corresponding methyl esters (1–20 mg) were lactonized in 0.5M HCl/CH_2Cl_2 1:4 (1 ml) overnight at 20°. The phases were separated, the CH_2Cl_2 phase was neutralized ($NaHCO_3$), dried (Na_2SO_4), and analyzed.

(±)-threo-*6-Hydroxydodecano-5-lactone* (*= (1'S,6R)-3,4,5,6-Tetrahydro-6-(1-hydroxyheptyl)pyran-2-one*; *(±)-threo-***7**). ^{1}H-NMR ($CDCl_3$): 2.34, 2.50 (2*m*, CH_2 (2)); 1.77–1.89 (*m*, CH_2 (3)); 1.59–1.77 (*m*, CH_2 (4)); 4.11 (*m*, CH (5)); 3.47 (*m*, CH (6)); 1.33–1.48 (*m*, CH_2 (7)); 1.12–1.33 (*m*, 4 CH_2); 0.77 (*t*, *J* = 7, Me(12)). ^{13}C-NMR: 171.77 (C(1)); 29.55 C(2); 18.16 C(3); 23.81 C(4); 83.07 C(5); 72.91 C(6); 32.50 C(7); 31.64; 28.99; 25.22; 22.35 (C(8)–C(11)); 13.84 C(12).

4. *Yeast Strain, Culture Conditions, Sampling and Workup:* Yeast (*S. cerevisiae* IfG-06136) was obtained from the culture collection of the Institut für Gärungstechnologie, Berlin. The organism was stored (4°) on wort agar slants. For metabolic experiments, cells were cultivated in a medium (200 ml, pH 5.5) 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), 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 culture medium, and 10 mg and 30 mg (±)-*threo*-(7,8-$^2H_2$)-**3**, 30 mg (5*R*,6*R*)-(5,6-$^2H_2$)-**4a**, 30 mg (5*S*,6*S*)-(5,6-$^2H_2$)-**4a**, 8 mg (±)-*threo*-(3,4-$^2H_2$)-**5**, 30 mg (9*R*,10*R*)-**10a**, and 30 mg (9*S*,10*S*)-**10a**, each in 250 µl EtOH, were immediately administered to different flasks. At certain times, *i.e.*, after 24, 48, 72, 96, 120, 144, 168, 216, 268, and 312 h, aliquots (10 ml) of the culture broth were taken, internal standards (5 ppm each of decan-1-ol, octano-5-lactone, and heptadecanoic acid) were added, the mixture was extracted with Et_2O (2 × 20 ml), the extract dried (Na_2SO_4), methylated with diazomethane, and converted to derivatives (silyl ether or trifluoroacetyl-ester) under standard conditions [6], if necessary. The quantification of γ-lactones by GC/MS rel. to the int. standard octano-5-lactone (method previously validated) was with a std. dev. of 10%. The hydroxylactones and dihydroxy fatty acid methylesters were analyzed *vs.* the 1-decanol standard (validated for 6-hydroxy-5-dodecanolactone (**7**) and 9,10-dihydroxyoctadecanoate (**10a**)) with a std. dev. of 15%.

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