Biosynthesis of Flavor Compounds by Microorganisms
6. Odorous Constituents of *Polyporus durus* (Basidiomycetes)*

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The neutral volatile constituents produced by shake flask cultured mycelium of *Polyporus durus* comprise aliphatic and aromatic alcohols, lactones and various carbonyl compounds, and sesquiterpenoids. The occurrence of five 2,3-unsaturated 4-olides (γ-lactones), some of which were identified for the first time in nature, is characteristic of the fungus. The presence of a synthetic triglyceride in the nutrient medium strongly favors the formation of lactones and other volatile flavor compounds.

Introduction

In microorganisms the production of sensorially active metabolites via anabolic and catabolic pathways is a widespread phenomenon. During the last decade, especially fungi were the aims of research, and some subjective odor impressions could be transformed into objective analytical data (ref. in [1, 2]). However, in most cases only surface cultures were investigated, and yields of volatiles rarely exceeded 1 $mg \cdot l^{-1}$.

Screening a number of species of the order Poriales the ability of a *Polyporus durus* strain to produce considerable amounts of 4-olides (= γ-lactones) in a liquid medium was recognized. As briefly mentioned in a previous paper [3], mass spectra pointed to the occurrence of a number of unknown volatiles with lactonic structure.

Lactones are of interest owing to their outstanding sensory (*e.g.,* 4-octanolide odor detection threshold in H_2O 7ppb [4]) and further physiological properties (*e.g.,* pheromone activity [5–7]). Therefore, efforts were undertaken to evaluate the structures of the fungal constituents and their biogenesis.

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Results and Discussion

Identification of volatile metabolites

Polyporus durus surface cultures grown on malt or yeast-malt agar exhibit a pleasant, but weak fruity-mushroom like odor. Upon growing in a liquid synthetic production medium a strong odor impression reminding of pineapple fruit and coconut is noted. An extract of the nutrient medium shows a complexity of volatiles which exceeds those of some flavor materials of plant origin (Fig. 1).

Among the compounds identified (Table I) are lower alcohols, 2-phenylethanol, and 3-octanone which are considered ubiquitous in fungi [8]. The nonpolar fraction I of the nutrient medium extracts contains a number of sesquiterpene hydrocarbons, the medium polar fraction II some oxygenated sesquiterpenes. Compounds of both groups occur in low concentrations ($< 10\ \mu g \cdot l^{-1}$) and, as shown by sniffing-capillary gas chromatography, do not possess pronounced sensorial qualities. Sesquiterpenes with mainly bicyclic skeletons have been reported in wood destroying fungi by several authors [1, 9].

The prominent compounds in the polar fraction III are 4-olides. They dominate the spectrum of volatiles quantitatively (Fig. 1) and with regard to the sensorial properties. Saturated 4-olides have been identified in fungi in a few cases only, *e.g.,* in cultures of *Pityrosporum* sp. [10] and in *Sporobolomyces odorus* [11, 12]. *Polyporus durus* produces the homologous 4-olides from four to ten carbons and in addition the homologous 2-en-4-olides from six to ten carbons, the 2-hepten-4-olide to 2-decen-4-olide being identified for the first time in a natural source. Some side

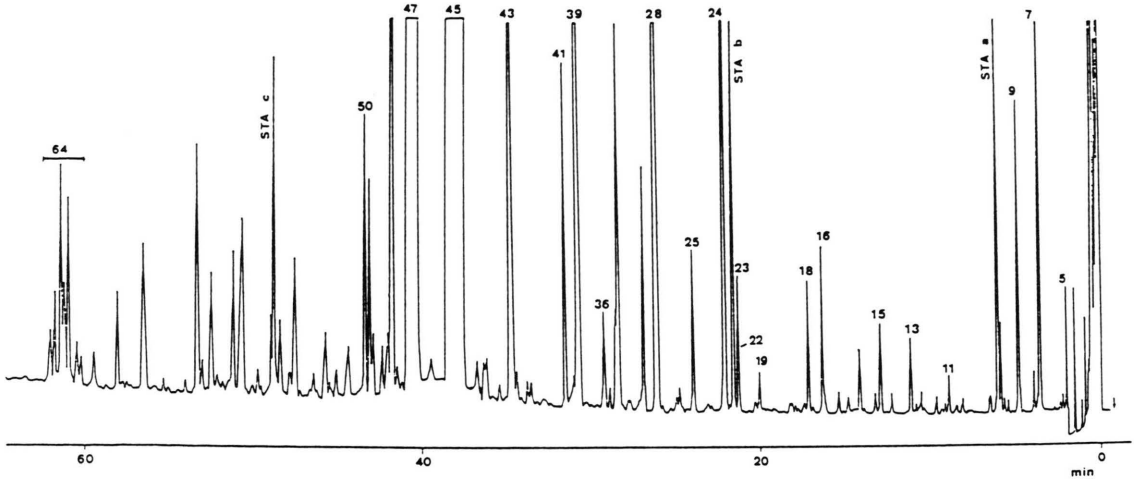

Fig. 1. Capillary GLC profile of volatiles produced by *Polyporus durus* (synthetic liquid nutrient medium, 200 rpm, incubation time 29 days). GLC conditions: column no. 1, attn. 64 – after 2 min attn. 16. Internal standards STA a = methyl heptanoate, STA b = methyl decanoate, STA c = methyl pentadecanoate, 1 mg $\times l^{-1}$ each. Peak numbers according to Table I.

Table I. Neutral volatile constituents produced by *Polyporus durus*.

Peak no.[a]	Compound	Fract.[b]	Conc.[c]	Ident.[d]	Odor[e]
	alcohols				
1	2-butanol	III	1	MS, RT	
2	1-propanol	III	1	MS, RT	
3	2-methyl-1-propanol	III	3	MS, RT	alcoholic
4	2-pentanol	III	1	MS, RT	
6	1-butanol	III	2	MS, RT	
7	3-methyl-butanol	III	4	MS, RT	alcoholic
8	3-methyl-3-buten-1-ol	II/III	1	MS, RT	
10	1-pentanol	II/III	2	MS, RT	
11	1-hexanol	II/III	4	MS, RT	sweet
12	3-*Z*-hexen-1-ol	II/III	1	MS, RT	
13	3-octanol	II	4	MS, RT	fruity
19	1-octanol	II	3	MS, RT	fruity-flowery
40	1-decanol	II	2	MS, RT	
46	2-phenylethanol	II	3	MS, RT	
53	3-phenylpropanol	II	2	MS, RT	
	ketones				
5	3-penten-2-one	II	2	MS, RT	
9	3-octanone	II	3	MS, RT	fruity
	esters[f]				
15	ethyl octanoate	II	3	MS, RT	estery
25	ethyl decanoate	II	3	MS, RT	estery
	lactones				
14	3-pentene-4-olide	III	2	MS, RT	
23	4-pentanolide	III	3	MS, RT	
24	4-butanolide	III	4	MS, RT	
28	4-hexanolide	III	4	MS, RT	fragrant
34	5-hexene-4-olide	III	2	MS, RT	
36	2-hexene-4-olide	III	3	MS, hydr.	
39	5-hexanolide	III	4	MS, RT	

Table I continued

Peak no.[a]	Compound	Fract.[b]	Conc.[c]	Ident.[d]	Odor[e]
41	4-heptanolide	III	3	MS, RT	fragrant
42	2-heptene-4-olide	III	2	MS, hydr.	fragrant
45	4-octanolide	III	5	MS, RT NMR	coconut
47	2-octen-4-olide	III	5	MS, NMR UV, hydr.	fruity coconut
48	5-octen-4-olide	III	2	MS, hydr.	coconut
49	6-octen-4-olide	III	4	MS, hydr.	coconut
50	4-nonanolide	III	3	MS, RT	fruity
55	2-nonene-4-olide	III	2	MS, hydr.	fruity
57	4-decanolide	III	1	MS, RT	peach
59	2-decene-4-olide	III	1	MS, hydr.	
62	4-methoxy-6-methyl-2-H-pyran-2-one	III	2	MS	
	sesquiterpenes				
17, 20, 21, 27	$C_{15}H_{24}$	I	1	MS	
26	*E*-β-farnesene	I	2	MS	
29	$C_{15}H_{22}$	I	1	MS	
30	γ-muurolene	I	2	MS	
31	β-bisabolene	I	2	MS	
32, 33	$C_{15}H_{24}$	I	3, 1	MS	
35	δ-cadinene	I	3	MS	
37	$C_{15}H_{24}$	I	1	MS	
38	$C_{15}H_{22}$	I	1	MS	
51, 60	$C_{15}H_{26}O$	II	1	MS	
61	$C_{15}H_{24}O$	II	2	MS	
63	farnesol	II	3	MS, RT	

[a] Peak numbers according to Fig. 1.
[b] Total extract was separated into three fractions on silica gel (see Experimental).
[c] Maximum concentrations of compounds during cultivation 1: $< 10\ \mu g \times l^{-1}$; 2: $10-100\ \mu g \times l^{-1}$; 3: $100-1000\ \mu g \times l^{-1}$; 4: $1-100\ mg \times l^{-1}$; 5: $> 100\ mg \times l^{-1}$.
[d] RT: retention time identical with reference compound on two different columns (no. 1/2 and no. 3, see Experimental). MS: mass spectrum identical with reference or literature data. hydr.: compound characterized via RT, MS upon Pd/C hydrogenation. NMR: spectrum identical with literature data. UV: absorbance maximum identical with literature data.
[e] Determined by sniffing-capillary GLC (see Experimental).
[f] Esters are not detectable in cultures grown without triglyceride.

chain unsaturated lactones accompany as in *Sporobolomyces* [11] their saturated analogues.

Many of the lactonic components give distinct odor impressions (Table I) and contribute to the characteristic flavor of the nutrient medium. Taking biological variations into account the presentation of concentration ranges in Table I has been given preference to exact concentration data for the single compounds.

Nutrient medium composition and lactone formation

A number of substrates which are thought to be involved in lactone biogenesis [11, 13, 14] have been added to growing cultures in separate experiments. Various fatty acids from two to eighteen carbons and glycerol are ineffective. Only the addition of the synthetic triglyceride Miglyol markedly enhanced the formation of lactones (Table II) and of most of the other metabolites listed in Table I. The Miglyol used is an odor- and colorless coconut oil fraction consisting of 56% octanoic, 42% decanoic, and < 2% dodecanoic acid (see Experimental). Independent of the presence of Miglyol the pH of the nutrient medium falls from initial 6.0 to below 3 after six days of cultivation. This low pH is a prerequisite for the stimulating effect of Miglyol. The formation of lac-

tones is strongly inhibited when the pH is controlled and kept within > 4.5 and 6.5 during the cultivation (Table II, sample F). The lactonization step, probably, takes place in the acid environment outside the cell, the 4-hydroxy acids being the real products of the fungal metabolism. This is supported by the occurrence of lactones in an acid fraction of the medium which, by a preceeding solvent extraction, has been quantitatively cleared of neutral compounds.

Table II. Effects of triglyceride in shake flask cultures of *Polyporus durus*.

Sample no. in Fig. 2	Triglyceride conc. [ml × 100 ml^{-1}]	Sum of lactones [mg × l^{-1}]	Residual glucose [%]	pH
A	1	168	71	2.5
B	1	281	49	2.4
C	1	225	0	2.5
D	–	10	52	2.3
E	–	23	0	2.3
F[a]	1	6	59	4.6

[a] 4.5 < pH < 6.5 by adding sterile NaOH during incubation.

Effect of triglyceride on cell protein

An attempt to get more insight into the mode of action of Miglyol has been made applying an isoelectric focusing technique to the soluble proteins of *Polyporus* (Fig. 2, Table II). Previously, investigations of fungal proteins using polyacrylamide electrophoresis were carried out for taxonomical classifications [15].

The patterns of soluble proteins of *Polyporus* change during the cultivation period (Fig. 2, A–C). Clear differences are further detectable between cultures of the same physiological age, but grown with or without the triglyceride supplemented to the nutrient medium (Fig. 2, B and D, C and E). On continued cultivation in the presence of Miglyol a number of major protein zones appear in the pI range > 5, while the stained zones at pI < 4.5 decrease. Similar distinct differences appear within the esterase isozyme patterns. An exception is sample no. F in Table II which in the presence of Miglyol is almost completely devoid of ester hydrolysing activities during the log phase (results not shown).

It is doubtful whether some of the new or enhanced protein zones are directly associated with lactone biogenesis: it is true the Miglyol drops disappear from the nutrient medium on prolonged incubation, but still a conversion rate of only ca. 0.3% can be calculated if a direct conversion of the acyl moieties into lactones is assumed. So the main routes of Miglyol metabolism do not lead to the volatiles described (Table I). Nevertheless, the correlation between the presence of the triglyceride and the altered cellular metabolism as expressed by the protein and esterase patterns is demonstrated.

Supplement of lipophilic phases

The Miglyol induced metabolic changes could be caused by physical properties of the triglyceride like influence on shear forces oxygen transfer, or adsorption to the hypen surface. Another effect could be a shift of metabolic equilibria in favor of the formation of the more lipophilic volatiles by accumulating them during certain periods of the cultivation. For this purpose Miglyol was successfully added to plant cell cultures [16, 17].

To parallel cultures of Polyporus Miglyol, paraf-

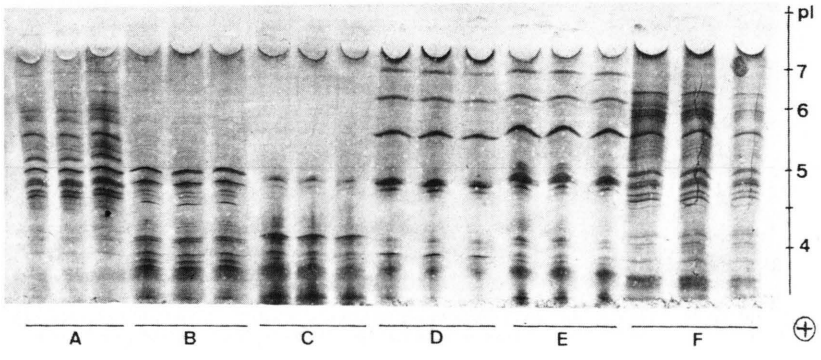

Fig. 2. Protein patterns of cell sap of *Polyporus durus* cultivated with (A, B, C, F) and without (D, E) a triglyceride. Isoelectric focusing on 50 µg polyacrylamide gel, separation distance 3 cm, protein conc. 100 ng per lane, Servalyt carrier ampholytes pH 3–7/3–10 (1:1, v/v), silver staining as in [31]. Sample numbers according to Table II.

fin, and Amberlite XAD 2 have been added. Amberlite XAD 2 is able to accumulate monoterpenes [18, 19] as well as lactones from a water phase. The results in extracts are shown in Fig. 3: neither Amberlite (bar c) nor paraffin (bar d) can substitute Miglyol with regard to lactone formation.

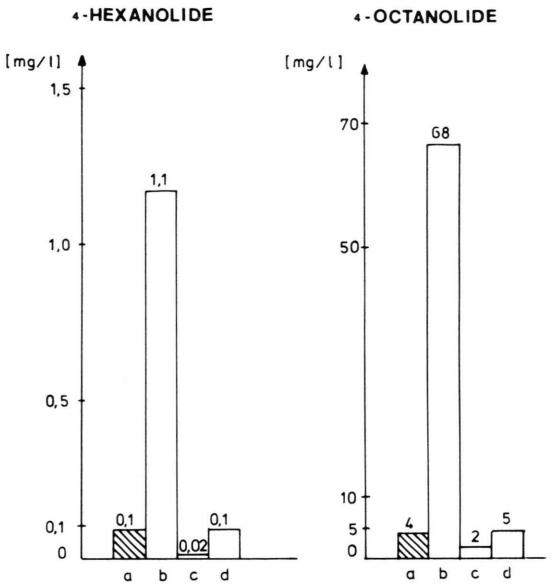

Fig. 3. Effect of lipophilic phases on lactone formation in shake flask cultivated (200 rpm) *Polyporus durus*. Addition of compounds after 23 days of cultivation (40% residual glucose), separation of volatiles after a total of 29 days. a: control, b: Miglyol (1 ml × 100 ml^{-1}), c: Amberlite XAD 2 (5 g × l^{-1}), d: paraffin (1 ml × 100 ml^{-1}).

Biogenesis of lactones

Numerous aliphatic and aromatic monocarboxylic acids, aliphatic dicarboxylic acids, keto − and hydroxy acids are found when the fungus is grown on a Miglyol containing nutrient medium. This indicates lipid hydrolysis and vigorous γ-, δ-, and ω-oxidation in excess of fatty acid substrate. Further examination of the acid fraction of the nutrient medium upon removal of the neutral compounds points to the direct involvement of the triglyceride into lactone biogenesis. Just those acid compounds, which would fit to a possible biogenetic scheme of lactone formation, reach maximum concentrations at time of maximum lactone formation (Table III). Both 4-oxo-octanoic acid (methyl ester *m/z* (%): 57 (100), 55 (72), 41 (55), 85 (52), 98 (45), 115 (37), 130 (28), 59 (25))

Table III. Time course of selected acid compounds (mg × l^{-1}, concentrations of methyl esters calculated from internal standard methyl heptanoate) in the triglyceride supplemented (1 ml × 100 ml^{-1}) nutrient medium of *Polyporus durus*.

Methyl esters	Cultivation time [days] 4	7	11	18	24	29
n-octanoate	0.2	0.6	0.5	1.3	0.6	0.3
n-decanoate	< 0.1	0.2	0.1	0.2	0.1	0.2
4-hydroxy-octanoate	−	< 0.1	0.1	0.8	1.7	0.4

and 4-hydroxy-octanoic acid (·/· *m/z* (%): 58 (100), 88 (40), 57 (38), 41 (28), 55 (19), 43 (18), 117 (17) ... 143 (1)) and the even numbered acyl moieties of Miglyol are present in nutrient medium extracts. The low actual concentrations may be explained by their role as intermediates in a highly active metabolism. The high yields of volatiles may be due to the fact that the fungus, not liberating more acyl moieties than to be metabolized, avoids toxic concentrations of free fatty acids.

It may be speculated at which point of time the double bond of the 2-alken-4-olides is introduced, *i.e.* prior or after hydratation of the acyl moiety. However, the formation of 2-octen-4-olide always precedes the formation of the saturated analogue.

A scheme for the lactone biogenesis according to these results is presented in Fig. 4. An active role of the triglyceride in the formation of lactones may be supposed, the lack of a well-working capillary radiogas chromatograph preventing a definite statement. Further work to evaluate the enantiomeric composition of the fungal lactones is in progress.

Experimental

Organism and culture conditions

Polyporus durus Jungh. (= *Osmophorus durus* (Jungh.) G. H. Cunn.) CBS 313.36, K. S. G. Cartwright was obtained from Centralbureau voor Schimmelcultures, Baarn, The Netherlands, and was maintained on 2% yeast-malt agar. The fungus was precultured on yeast-malt solution and allowed to grow for three weeks at 27 °C and natural light regime. Subsequently, one ml of the homogenized mycelial mat was inoculated into 100 ml of a synthetic glucose-asparagine-mineral salt medium [20] as described in [21]. This "production" medium contained

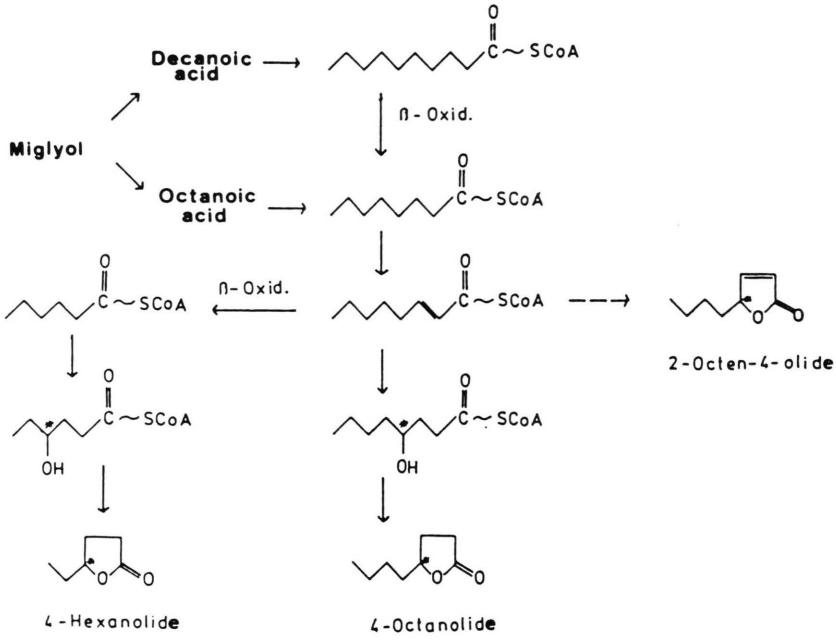

Fig. 4. Proposed scheme of lactone biogenesis in *Polyporus durus* cultures.

1 ml sterile filtered (Millex-GS, Millipore) Miglyol 812 neutral fat (Dynamit-Nobel), unless otherwise indicated. The shake flasks were cultivated at 200 rpm, 27 °C, and natural light regime. All other supplements were added as sterile filtrates. Autoxidative formation of volatiles was controlled by taking along identical, but mycelium-free flasks during each experiment.

Isolation of metabolites

Lipid containing nutrient broth was steam distilled (400 ml = 1 h), the distillate diluted with 100 ml 2% $NaHCO_3$, internal standards added, and the solution extracted three times with pentane: dichloro methane (2:1). The combined extracts were dehydrated over anhydrous Na_2SO_4, concentrated on a Vigreux column at 40 °C [22], and fractionated by descending column chromatography on silica gel according to [23]. Three fractions were eluted with 200 ml pentane (I), followed by 200 ml pentane: diethyl ether (100:7.5, II), and 200 ml diethyl ether (III), and concentrated as above. Supplemented paraffin elutes completely in fraction I.

Lipid-free nutrient broth was separated from mycelium by filtration, and, after dilution with $NaHCO_3$, treated as described above for steam distillate. Steam distillation induced changes of compounds as compared to directly extracted samples were not detected in controls.

Acid compounds were separated from the clear nutrient broth by a procedure as follows: neutral compounds and residual lipid were quantitatively extracted at pH 8 with pentane: dichloro methane (2:1), and the acidified water phase (4n HCl) extracted with diethyl ether at pH 1.5. The combined etheral phases were reextracted with 2% $NaHCO_3$ at pH 8, the water phases again acidified, extracted with ether, dried and concentrated as above. Methylation of carboxylic acids was performed with diazo methane [24].

Miglyol was re-esterified with BF_3/MeOH [25].

Analysis of volatiles

Gas chromatographic analyses were run with a Carbowax 20 M glass capillary column (25 m × 0.25 mm i.d., WGA, no. 1), a Carbowax 20 M fused silica column (25 m × 0.3 mm i.d., crosslinked, made by Dr. G. Leupold at the institute, no. 2 for free and methylated acid compounds only), an OV 101 glass capillary column (50 m × 0.32 mm i.d., Jaeggi, no. 3), and a Carbowax 20 M SCOT column (40 m × 0.5 mm i.d., WGA, no. 4 for sniffing GLC only). General conditions were carrier gas H_2, linear temperature

Table IV. Mass spectra (70 eV) of unsaturated lactones of *Polyporus durus* nutrient broth.

Peak no. in Table I	*m/z* [%]
36	83 (100), 55 (43), 57 (17), 39 (15), 84 (13), 54 (9), 56 (6), 41 (5)
42	55 (100), 43 (88), 41 (79), 84 (64), 97 (56), 39 (49), 83 (30), 71 (17)
47	55 (100), 41 (94), 84 (91), 111 (42), 57 (38), 39 (37), 83 (31), 85 (22)
48	111 (100), 55 (67), 41 (63), 39 (61), 56 (59), 85 (27), 81 (26), 83 (24)
49	85 (100), 57 (15), 39 (14), 55 (13), 41 (7), 56 (4), 43 (3)
55	43 (100), 55 (58), 84 (53), 41 (43), 39 (25), 125 (13), 83 (12), 71 (9)
59	43 (100), 55 (43), 39 (41), 84 (28), 57 (26), 39 (12), 69 (6), 73 (3)

programs and flame ionization detector. A mass spectrometer, Finnigan 4021 (quadropole), was directly coupled with a 9610 gas chromatograph, equipped with a Carbowax 20 M glass capillary column (25 m × 0.25 mm i.d., Mega, no. 5). Details of operations are given in [26, 27].

Mass spectra of the unsaturated lactones of Polyporus are compiled in Table IV. All of these compounds disappear from the mixture of volatiles in fraction III upon hydrogenation with Pd/C, while the concentrations of corresponding saturated lactones increase to the same extent.

Qualitative analyses were carried out by means of GLC retention on different columns, sniffing-GLC, and mass spectral data of authentic reference compounds and literature data.

UV spectra of pure 4-octanolide and a mixture of 4-octanolide and 2-octen-4-olide (5:1), obtained from fraction III and redissolved in MeOH, were recorded with a Beckman Mod. 25. The absorption maximum of the mixture at 217 nm points to a conjugated double bond in agreement with spectra of 2,3-butenolides [28].

^{1}H NMR spectra of both samples in $CDCl_3$/TMS were recorded with a Hitachi R 24, 60 MHz. Pure 4-octanolide shows no bands at > 4.8 ppm. The difference spectrum of the mixture at > 4.8 ppm is: 4.90–5.20 (m, 1H), 6.12 (d, d, 1H), 7.51 (d, d, 1H) as for synthetic 2-octen-4-olide [28, 29].

Analysis of proteins

Disruption of cell material with a BIOX-X-press, purification of proteins, preparation of ultrathin-layer polyacrylamide gels, silver staining of proteins, and visualization of esterase were described previously [30, 31].

Analysis of medium parameters

Glucose determinations were carried out enzymatically (Boehringer) using a Shimadzu Mod. UV-120-02. Measurement of pH with a glass electrode/Knick 530 pH meter.

All chemicals were p.a. or "biochemistry" quality; solvents were redistilled before use.

Acknowledgements

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