



# Biogenesis of spiroketals by submerged cultured basidiomycete *Trametes hirsuta*

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## Abstract

Volatile spiroketals are well-documented semiochemicals secreted by beetles and wasps for the intra- and interspecies communication. Its use in insect traps and as natural herbicide makes them of commercial interest. Besides insects, fungi are well-known producers, but the fungal biogenesis of spiroketals has remained speculative. Product formation along fatty acid degradation based on non-labeled feeding experiments was assumed. Thus, the observed occurrence of conophthorin and (E)- and (Z)-chalcograns in submerged cultures of the basidiomycete *Trametes hirsuta* prompted a precursor study aiming at a more detailed insight into their formation. Supplementation of (9Z,12Z)-octadecadienoic (linoleic) acid resulted in elevated product yields and the identification of a fourth spiroketal, 2,8-dimethyl-1,7-dioxaspiro[5.5]-undecane. However, no intermediates of fatty acid degradation suitable as spiroketal precursors were identified. In addition, the hyphae lacked lipoxygenase activity, which was formerly supposed to be mandatory for spiroketal formation. Supplementation of 1-/2-<sup>13</sup>C acetate showed incorporation of the label into chalcogran. Therefore, a formation along the polyketide pathway analogous to insects was concluded.

**Keywords** Spiroketals · Chalcogran · Conophthorin · *Trametes hirsuta* · Polyketide

## Introduction

Volatile spiroketals comprise an important group of semiochemicals isolated from prokaryotes and eukaryotes from marine and terrestrial environments (Booth et al. 2009). More than 30 structures have been identified. All structures ascribe to five basic ring systems, namely, 1,6-dioxaspiro[4.4]nonane (1), 1,6-dioxaspiro[4.5]decane (2), 1,6-dioxaspiro[4.6]undecane (3), 1,7-dioxaspiro[5.5]undecane (4), and 1,7-dioxaspiro[5.6]dodecane (5) (Fig. 1). The majority of insect spiroketals, cyclic ketals in which the ketal carbon is the only

common atom of two rings, contains up to 13 odd-numbered and in part alkylated carbon atoms (Booth et al. 2009). The most common structures are C<sub>11</sub>-spiroketals such as 2,8-dimethyl-1,7-dioxaspiro[5.5]-undecane (8), but also C<sub>9</sub> bodies occur. Especially 2-ethyl-1,6-dioxaspiro[4.4]nonane (6), called chalcogran, and 7-methyl-1,6-dioxaspiro[4.5]decane (7), called conophthorin, play a key role in the intra- and interspecies communication of insects (Zhang et al. 2002; Booth et al. 2009; Francke et al. 1995). These compounds typically act as sex pheromones but also participate in insect-plant communication (Zhang et al. 2002; Beck et al. 2012).

Besides insects and plants, fungi are also known to produce spiroketals. Although the specific mechanisms differ depending on insect life strategy and fungal species, the ecological role of this association is well understood (Kandasamy et al. 2016). Beetles often inoculate a tree with fungal spores, because fungi play an important role in insect nutrient supply and the degradation of host defenses (Hammerbacher et al. 2013; Bentz and Six 2006; Krokene and Solheim 1998). The majority of spiroketal-producing fungi was assigned to the phylum of *Ascomycota*, but some *Basidiomycota* are also known (Kandasamy et al. 2016). Despite this well-described ecological niche, data on biosynthetic pathways are rare. According to Beck et al., conophthorin may be produced in

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Miriam Grosse had done the experiment at the old institution and has now moved to a new one.

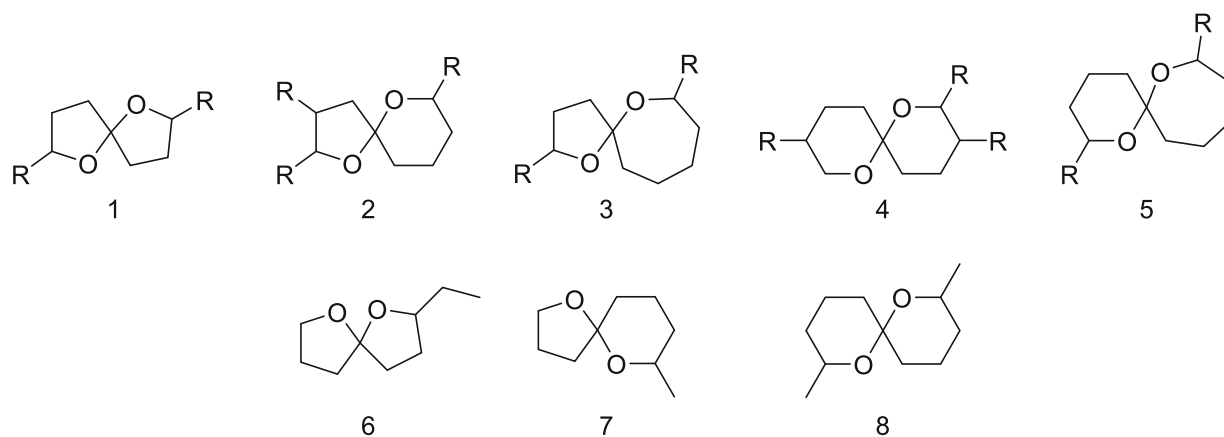
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**Fig. 1** Spiroketal ring systems. Different substituents lead to a large variety of volatile products

fungi along linoleic acid degradation. Conophthorin formation is initiated by a lipoxygenase (LOX)-catalyzed oxidation, accounting for cleavage products, such as hexan-1-ol and the corresponding  $C_{12}$  carboxylic acid. During routine screenings, spiroketals were identified in submerged cultures of the basidiomycete *Trametes hirsuta*, a saprobiontic white-rot fungus. As growing cell cultures enable controlled precursor supplementation and metabolite sampling on larger scales, the fungus served as a model organism to study the biochemical pathway of spiroketal formation in higher fungi.

## Materials and methods

### Chemicals and substances

If not stated otherwise, all media components and chemicals were purchased from Carl Roth (Karlsruhe, Germany) and Merck (Darmstadt, Germany). Linoleic acid, 2-amino-2-methyl-propanol, and labeled sodium acetate ( $1-^{13}C$  and  $2-^{13}C$ ) were ordered from Sigma-Aldrich (St. Louis, Missouri, USA). (E)-Conophthorin ((5*S*,7*S*)-7-methyl-1,6-dioxaspiro[4.5]decane) was purchased from ChemTica International S.A. (Santo Domingo, Costa Rica), and chalcogran was purchased by Sigma-Aldrich (St. Louis, Missouri, USA). Solvents were purified by in-house distillation.

### Cultivation conditions

*Trametes hirsuta* [German Collection of Microorganisms and Cell Cultures GmbH, DSMZ (Braunschweig, Germany) no. 7052] was grown in submerged cultures using standard nutrient liquid (SNL) (30.0 g/L glucose monohydrate, 4.5 g/L L-asparagine monohydrate, 3.0 g/L yeast extract, 1.5 g/L  $KH_2PO_4$ , 0.5 g/L  $MgSO_4$ , 5  $\mu g/L$   $CuSO_4 \cdot 5 H_2O$ , 80  $\mu g/L$   $FeCl_3 \cdot 6 H_2O$ , 30  $\mu g/mL$   $MnSO_4 \cdot H_2O$ , 90  $\mu g/mL$   $ZnSO_4 \cdot 7 H_2O$ , 400  $\mu g/mL$  EDTA) and minimal medium at pH 6.0 (MM; 3.0 g/L glucose monohydrate, 1.0 g/L yeast extract, 1.5 g/L  $KH_2PO_4$ , 0.5 g/L

$MgSO_4$ , 5  $\mu g/L$   $CuSO_4 \cdot 5 H_2O$ , 80  $\mu g/L$   $FeCl_3 \cdot 6 H_2O$ , 30  $\mu g/mL$   $MnSO_4 \cdot H_2O$ , 90  $\mu g/mL$   $ZnSO_4 \cdot 7 H_2O$ , 400  $\mu g/mL$  EDTA; pH 6.0).

Fungal growth was maintained on SNL agar using SNL as described above with additional 15 g/L agar-agar. Pre-cultures of 150 mL SNL were inoculated with 1  $cm^2$  agar and homogenized. After 7 days at 150 rpm and 24 °C on a rotary shaker (Infors, Bottmingen, Switzerland), main cultures of 250 mL SNL were inoculated with washed mycelium from 25 mL pre-culture. Main cultures were grown, if not stated otherwise, without additives. As additives, 5 % (v/v) linoleic acid and 12 mM labeled or non-labeled sodium acetate were selected.

### Volatile analysis

Main cultures were extracted by sequential stir bar sorptive extraction (SBSE) using Twisters (10 mm  $\times$  0.5 mm, Gerstel, Mülheim, Germany) coated with polydimethylsiloxane (PDMS) as described elsewhere (Grosse et al. 2020; Sampedro et al. 2009). In brief, 5 mL of the respective broth were extracted for 1 h at room temperature. Afterwards, 30% (w/v) sodium chloride was added and a second extraction step was performed with another stir bar. After extraction, stir bars were rinsed with bidistilled water, dried, and stored at 4 °C until analysis. Both stir bars were analyzed together by means of thermal desorption gas chromatography.

Qualitative and quantitative analyses were performed using an Agilent 6890N (Agilent Technologies, Santa Clara, CA, USA) equipped with a thermal desorption unit and a CIS 4 injection system (Gerstel, Mülheim, Germany). Twister desorption: TDS 3, 20 °C for 3 min, 60 °C/min to 230 °C held for 2 min, splitless mode; CIS 4 with a liner filled with Tenax TA, -10 °C, 10 °C/s to 230 °C held for 2 min, solvent vent splitless mode (Gerstel, Mülheim, Germany). Samples were separated on a polar HP Innowax column (30 m, 0.25 mm, 0.25  $\mu m$ , Agilent J&W GC Columns, Santa Clara, CA, USA). After separation (40 °C for 3 min, 6 °C/min until 230 °C for 10 min), the effluent was split 1:1 to an olfactory detection port

(ODP3, Gerstel, Mülheim, Germany) and an Agilent 5977A mass selective detector (interface 230 °C, ion source 200 °C, quadrupole 100 °C, electron impact ionization 70 eV, scan range  $m/z$  33–500 amu; Agilent Technologies). Retention indices according to Van den Dool and Kratz were calculated using homologue alkanes from C<sub>8</sub> to C<sub>30</sub>.

For (semi)quantitation, external calibration was performed using five aqueous chalcogran solutions in the range from 0.5 to 2.5 mg/L. Respective standards were prepared from five 500-fold concentrated stock solutions (250–625 mg/L) prepared in methanol. Calibration standards were produced by adding 10- $\mu$ L stock solution to 5 mL of pure water. Extraction and analysis were performed as described above.

### Fatty acid analysis

Main cultures were grown as described above with linoleic acid supplementation. For each analysis, a separate main culture was inoculated and harvested at different days (3, 4, 8, 9, and 10). The mycelium was separated from the culture *via* vacuum filtration (0.45  $\mu$ m). The lyophilized mycelium (Alpha-1-4 LSCbasic, Martin Christ GmbH, Osterode am Harz, Germany) was extracted with the azeotropic mixture of pentane/diethyl ether (1:1.12 *n/n*). Extracts were concentrated to approximately 1–2 mL using Vigreux rectification. Residual solvent was carefully removed under nitrogen and re-dissolved in 200  $\mu$ L dichloromethane. Aliquots of the samples were derivatized using two different methods for qualitative analyses.

For 4,4-dimethyl-oxazoline (DMOX) derivatization, 50- $\mu$ L sample was mixed with 500  $\mu$ L of 2-amino-2-methyl-propanol (AMP) to create an excess of substrate. The mixture was left to react overnight at 100 °C. After incubation, samples were cooled to room temperature. Afterwards, dichloromethane was added to dissolve all crystals in the reaction tube that were formed during incubation. Residual AMP was removed by three washing steps with pure water. The washed organic fraction was dried over sodium sulfate prior to analysis. As positive control, appropriate concentrated linoleic acid solutions were used.

For silylation, a sample to agent ratio of 1:5 was used. Hexamethylsilane, trimethyl-chlorosilane, and pyridine were used in a ratio of 3:1:9. Reaction mixtures were incubated for 30 min at 70 °C. Prior to chromatographic analysis, samples were dried overnight using anhydrous sodium sulfate.

After derivatization, samples were analyzed on a 7890B GC-system (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 5977A MS detector (interface 230 °C, ion source 200 °C, quadrupole 100 °C, electron impact ionization 70 eV, scan range  $m/z$  33–500 amu; Agilent Technologies). As stationary phase, DB-5MS UI (30 m, 0.25 mm, 0.25  $\mu$ m, Agilent J&W GC Columns) was selected. Samples of 0.5  $\mu$ L were analyzed with the following

temperature program: 40 °C for 3 min, 6 °C/min until 230 °C, 25 °C/min until 325 °C, and finally hold for 10 min.

### Lipoxygenase activity

For the detection of intracellular LOX activity, 0.2 g wet mycelium of *Trametes hirsuta* was disrupted with a Precellys 24 bead mill (PEQLAB, Erlangen, Germany) in 500  $\mu$ L 50 mM sodium phosphate buffer (pH 6.0). The lysate was centrifuged and the intracellular enzymes immediately used for activity testing.

Fifty microliters of enzyme solution was mixed with 290  $\mu$ L buffer in a photometric plate. The lysate and the culture supernatant were analyzed. The reaction was initiated by addition of 10  $\mu$ L 2 mM linoleic acid emulsion (20  $\mu$ L linoleic acid, 20  $\mu$ L Tween, 60  $\mu$ L sodium hydroxide filled up to 2 mL with distilled water). The linoleic acid conversion to the conjugated hydroperoxydienes was measured for 20 min at 234 nm (Synergy 2, Biotek, Bad Friedrichshall, Germany). As blanks, the enzyme solution was replaced with buffer. Enzymatic activity was calculated with the molar coefficient of the reaction product ( $\epsilon = 2.5 \cdot 10^4$  /M /cm). Each measurement was performed in triplicates.

## Results

### Identification of the produced spiroketals

Four spiroketals, namely, conophthorin, (E/Z)-chalcogran, and 2,8-dimethyl-1,7-dioxaspiro [5.5]undecane, were identified in submerged cultures of *T. hirsuta* by means of gas chromatography (retention index) mass spectrometry (spectral library). Conophthorin and E/Z-chalcogran were additionally confirmed using authentic references (Table 1). Based on the published elution order (Beck et al. 2012), the chalcogran isomers were assigned as shown in Table 1.

The fourth spiroketal, identified as 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, was detected in low concentrations in the culture supernatant after linoleic acid supplementation, only. Although a reference compound was not available, the fragmentation pattern of the electron impact (EI) spectrum of this spiroketal matched well with spectral libraries (NIST, Wiley) and is thus considered identified.

### Production of spiroketals

Conophthorin and chalcogran were found in the SNL medium of *T. hirsuta* as genuine metabolites without any further additives. The two diastereomeric chalcograns were detected in a nearly constant ratio of 60:40 (E:Z) over time. As shown in Fig. 2, after a 3-day lasting lag-phase, the concentration of conophthorin rose constantly to an intermediate plateau (days 6 to 8, 0.05 mg/L)

**Table 1** Spiroketal identified in liquid cultures of *T. hirsuta*

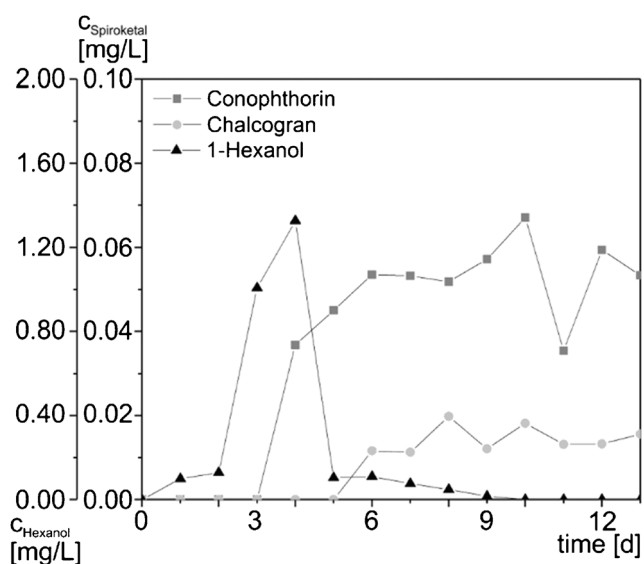
Spiroketal	Odor impression	RI	RI <sub>Lit.</sub>	Identified
(Z)-Chalcogran (6)	Herbal, fruity, fresh, rotten Citrus fruits	1465	1348	A, B, C,
[(Z)-2-Ethyl-1,6-dioxaspiro[4.4]nonane]				
(E)- Chalcogran (6)	Herbal, fruity, fresh, rotten Citrus fruits	1473	1353	A, B, C
[(E)-2-Ethyl-1,6-dioxaspiro[4.4]nonane]				
(E)-Conophthorin (7)	Fruity (sweet), lemon peel, rotten, hay, vanilla	1391	1280	A, B, C
[(E)-7-Methyl-1,6-dioxaspiro[4.5]decane]				
2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane <sup>1</sup> (8)	Herbal, grassy, woody, green, moldy, fruity	1657	-	B <sup>2</sup>

<sup>1</sup> Solely identified in cultures supplemented with linoleic acid supplement. <sup>2</sup> Compound tentatively identified. A, retention index on a non-polar phase; B, comparison of EI mass spectra with data bases (NIST, Wiley); C, reference substance

and reached a maximum concentration of 0.07 mg/L on day 10. Afterwards, the concentration dropped rapidly. In contrast, chalcogran generation started 2 days later and remained on an unsteady level of  $0.015 \pm 0.005$  mg/L from day 7 on.

According to Beck et al., spiroketals of *T. hirsuta* may be produced along linoleic acid degradation (2012). To validate this hypothesis for *T. hirsuta*, submerged cultures were supplemented with linoleic acid. Results displayed in Fig. 3 show conophthorin production boosted by a factor of 20, whereas chalcogran concentration even increased by a factor of 100. In addition, 1-hexanol, a supposed cleavage product of linoleic acid hydroperoxide, was also detected in higher concentrations. Additionally, 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, which was not present in non-supplemented cultures, was detected in the liquid culture medium.

Semi-quantitative analyses showed that observable conophthorin production started on day 4, the day when production of 1-hexanol peaked (Fig. 2). So far, the data obtained seemed in accordance with a hypothetical pathway *via* fatty acid degradation.



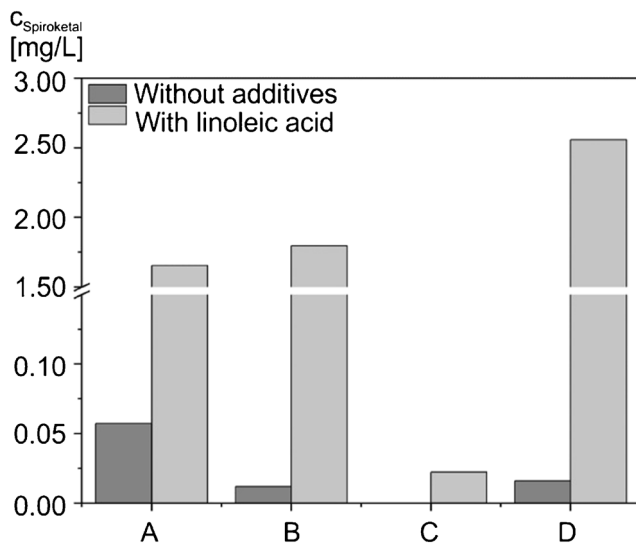
**Fig. 2** Time-dependent production of the main spiroketals by *T. hirsuta* in submerged cultures without supplements (SNL medium)

To add more evidence, the identification of intermediate degradation products of linoleic acid was attempted. Fungal mycelium was harvested at days 3, 4, 8, 9, and 10, and the fatty acids were extracted and derivatized. Additionally to silylation, DMOX derivatization was performed to locate the position of double bonds. Non-derivatized samples were evaluated to identify possible hydroxyketone precursors of the respective spiroketals. Apart from 1-hexanol, none of the expected intermediates was detected. LOX activity, which is believed to form 1-hexanol, was monitored during cultivation. In contrast to expectations, LOX activity was neither found in disrupted cells nor in the culture supernatant. This finding was crosschecked with genomic data of *T. hirsuta*. No lipoxygenase genes were identified through a NCBI BLAST search (April 23, 2020). In conclusion, spiroketals of *T. hirsuta* are most likely not produced by a LOX-initiated degradation of linoleic acid. An alternative pathway has to exist. Investigations with insects showed incorporation of labeled acetate, propanoate, malonate, succinate, and glutamate into 1,7-dioxaspiro[5.5]undecane, indicating that spiroacetals were synthesized *de novo* rather than by the metabolism of a dietary compound (Booth et al. 2009).

## Biogenesis of spiroketals

Since linoleic acid supplements boosted spiroketal yields, its  $\beta$ -oxidation product, acetyl-CoA, may serve as a building block for *de novo* biogenesis, possibly along the polyketide pathway, as was described for insects. To verify this, a surplus of acetate was provided via supplementation of  $1\text{-}^{13}\text{C}$ - or  $2\text{-}^{13}\text{C}$ -labeled sodium acetate to trigger the polyketide pathway. Products of the polyketide pathway were expected to incorporate at least parts of the surplus of labeled acetate directly or *via* malonic acid. Accordingly, this would result in changed isotopic patterns of the molecular or fragment ions thereof in the electron impact mass spectrum of the respective spiroketal.

Due to the lower abundance of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and [(E)-7-methyl-1,6-dioxaspiro[4.5]decane], the intensity of the chalcogran mass spectrum was qualified for a reliable evaluation of the isotopic



**Fig. 3** Impact of linoleic acid addition on the product concentration. (SNL medium, harvest on day 9). A, 2-ethyl-1,6-dioxaspiro[4.4]nonane (chalcogran); B, 7-methyl-1,6-dioxaspiro[4.5]decane (conophthorin); C, 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane; D, 1-hexanol

pattern. As the signal of the molecular ion at  $m/z$  156 was less abundant, a valid evaluation of the isotopic pattern was based on the most intense fragment ion at  $m/z$  127 ( $M-C_2H_5$ ) in the EI mass spectrum of chalcogran (Fig. 4). When fed with  $^{13}C$ -labeled acetate, the fungus produced chalcogran possessing a significantly extended isotopic pattern. Supplementation with  $1-^{13}C$  acetate resulted in a significant intensity shift of the  $^{13}C$  isotopic peak ( $m/z$  128) from 7.2 (accounting approximately for seven natural carbon atoms, 7.7% calc.) to 16.9% and for supplemented  $2-^{13}C$  to 21.2%. Three additional isotopic mass peaks were detected indicating the incorporation of more than one labeled acetate unit. This finding strongly advises chalcogran as a *de novo*-synthesized and polyketide-derived metabolite of *T. hirsuta*. Miethbauer et al. showed that rubellins were biosynthesized in an ascomycete *via* the polyketide pathway by demonstrating the incorporation of both  $1-^{13}C$  and  $2-^{13}C$  acetate (Miethbauer et al. 2006).

## Discussion

### Occurrence of the produced spiroketals

Conophthorin is a known repellent secreted by several plants to defeat parasites, inter alia the navel orange worm, a common parasite of pistachios (Huber and Borden 2001; Beck et al. 2012). It was also identified as a specific volatile for workers of social wasps like *Paravespula vulgaris* and as a male specific pheromone of the bark beetle *Leperisinus fraxini* (Francke et al. 1978). Typically, the (E)-isomer is present in biological systems (Zhang et al. 2002) as in the culture supernatant of *T. hirsuta*. (E)- and (Z)-chalcogran are known

natural semiochemicals and were found among other sex pheromones in several male bark beetles (Booth et al. 2011). *Pityogenes chalcogruphus*, for instance, a bark beetle causing a pest of Norway spruce, secretes both isomers as a such (Birgersson et al. 1990). Various fruit flies including *Dacus cucumis* and *D. halfordiae* secrete this 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane as pheromone for sexual attraction (Kitching et al. 1989; Noushini et al. 2020). However, no commercial interest for this compound arose, yet.

Especially conophthorin and the two chalcogran isomers were the focus of further studies, because both are of commercial interest (Huber and Borden 2001). Concurrent production of both spiroketals is rare in nature, but was reported for fungal spores of the ascomycetes *Aspergillus flavus*, *A. niger*, *A. parasiticus*, *Penicillium glabrum*, and *Rhizopus stolonifer* (Beck et al. 2012).

### Biogenesis of spiroketals

Important bioactive natural products are produced along the polyketide pathway in fungi. Iterative Claisen condensations of small carboxylic acid derivatives, so called polyketide building blocks, form linear polyketo chains. The most prevalent starter units are CoA-thioesters of acetic, malonic, and methyl malonic acid. Depending on the respective polyketide synthases, condensation reactions are followed by concurrent reductive modification of internal keto groups and/or the terminal CoA-ester. Hydrolysis of the latter releases free  $\beta$ -keto acids that are prone to spontaneous decarboxylation to give the corresponding methyl ketones. Together with several  $C_3$ -starter units, decarboxylation opens the route to odd-numbered small and volatile polyketides. Polyketides in fungi are formed by different polyketide synthases (type I, II, or III) via essentially the same mechanisms, but some or even all of the steps of the reductive loop can be omitted. In combination with available starter units, a respectable number of diverse spiroketals can be generated. Based on the general biochemical steps of the polyketide synthesis, a hypothetical pathway for each of the spiroketals of *T. hirsuta* was proposed (Figs. 5, 6, 7).

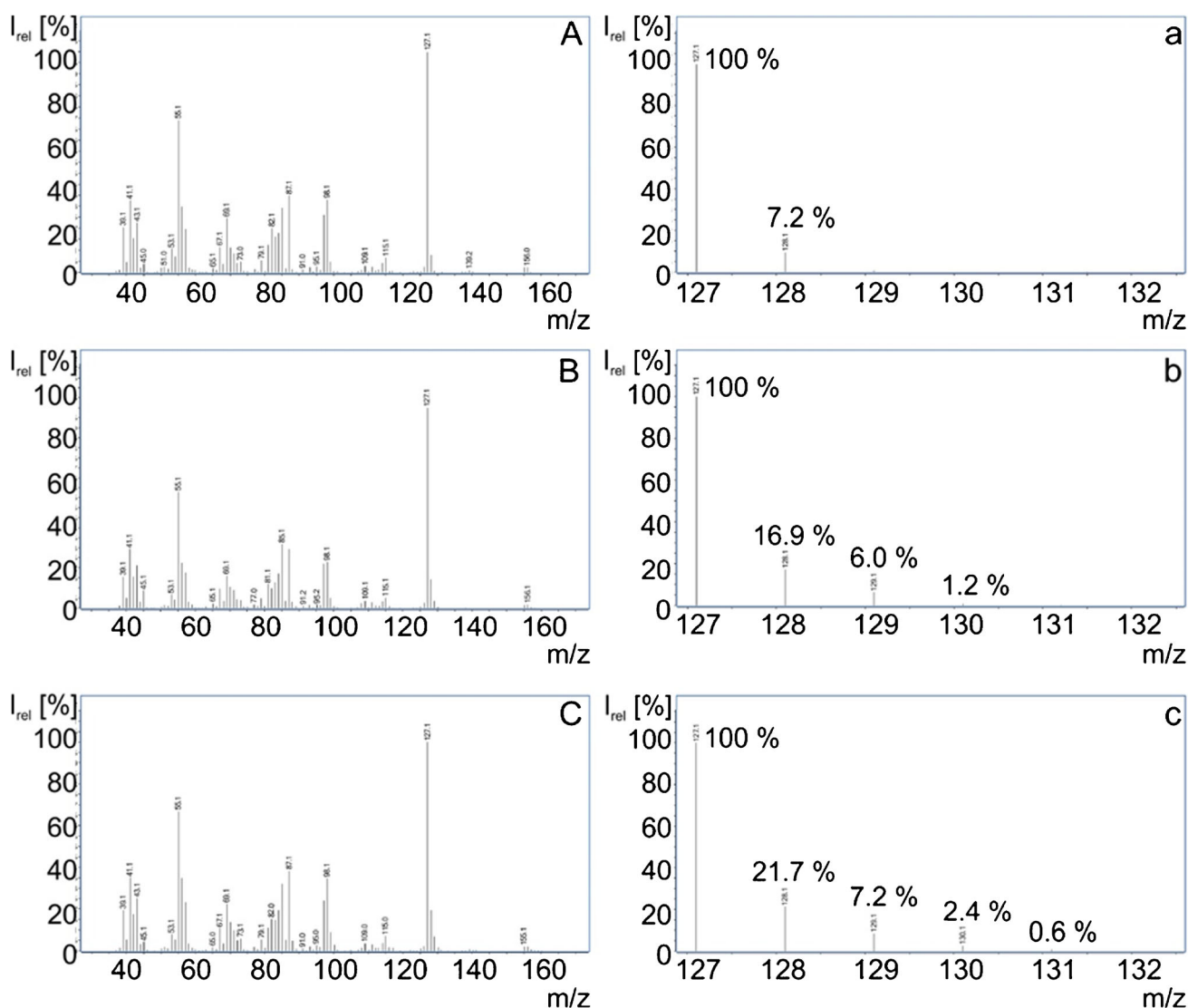
Formation of the symmetric 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (8) can be explained by condensation of five malonyl-CoA units to an acetyl-CoA starter unit under decarboxylation and concurrent partial reduction of the resulting polyketo chain (Fig. 5). After hydrolysis of the resulting thioester (13), the released 3,11-dihydroxy-7-ketododecanoic acid undergoes spontaneous decarboxylation to (14) and finally, after reduction of the methyl ketone group, yields the direct precursor 2,10-dihydroxy-6-undecanone (15). The mechanism of the thermodynamically favored double cyclisation to the respective spiroketal is shown exemplarily for chalcogran (Fig. 7).

Conophthorin consists of two heterocyclic rings, a methylated 4H-pyran and an unsubstituted 4H-furan, which are

bridged via one shared carbon atom. In contrast to 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (8), the same simple iterative condensation of malonyl-CoA units to a C<sub>2</sub>-starter unit and concurrent reduction steps, however, cannot explain the formation of this spiroketal. The unsubstituted 4H-furan requires a terminal hydroxyl group and a keto group at C<sub>4</sub>, which cannot be furnished by the reduction of one of the internal keto groups of the iterative extension of C<sub>2</sub>-units to a C<sub>2</sub>-starter unit. However, the required primary alcohol can be obtained through reduction of the thioester group. Thus, hydrolysis and decarboxylation of the respective acyl-CoA need not take place. However, this hypothesis calls for a different starter unit than acetyl- or malonyl-CoA. Pyruvyl-CoA provides both the odd-numbered starter unit required to end up with a C<sub>9</sub> carbon chain and the oxo-functionality at C<sub>2</sub>, which

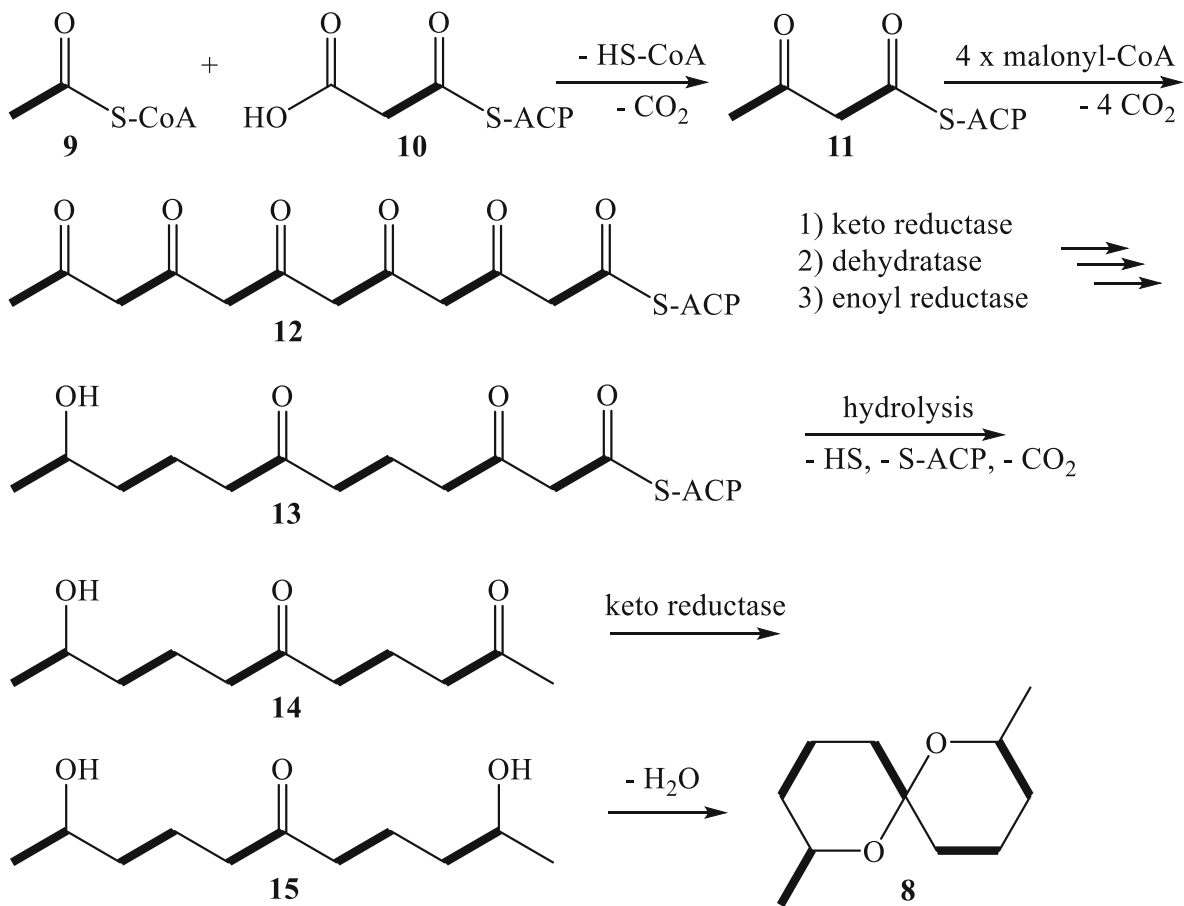
is essential for the formation of a methyl 4H-pyran ring. Additionally, proper cyclisation requires a formal shift of an internal keto group as shown in Fig. 6. This can be accomplished by an incomplete reduction of an internal keto group to a C-C double bond. Rehydration of this intermediate double bond and subsequent oxidation of the resulting alcohol will complete the shift of the keto group. Finally, the dihydroxy-keto-precursor molecule (19) cyclizes to conophthorin, as shown in Fig. 7.

Biosynthesis of chalcogran is partially similar to conophthorin formation. The formation of the unsubstituted 4H-furan follows the same route. However, the ethyl-substituted second 4H-furan ring would require methyl malonyl-CoA as starter unit, which would result in the terminal ethyl group after decarboxylation. Following the biosynthetic route depicted in Fig. 7, at least



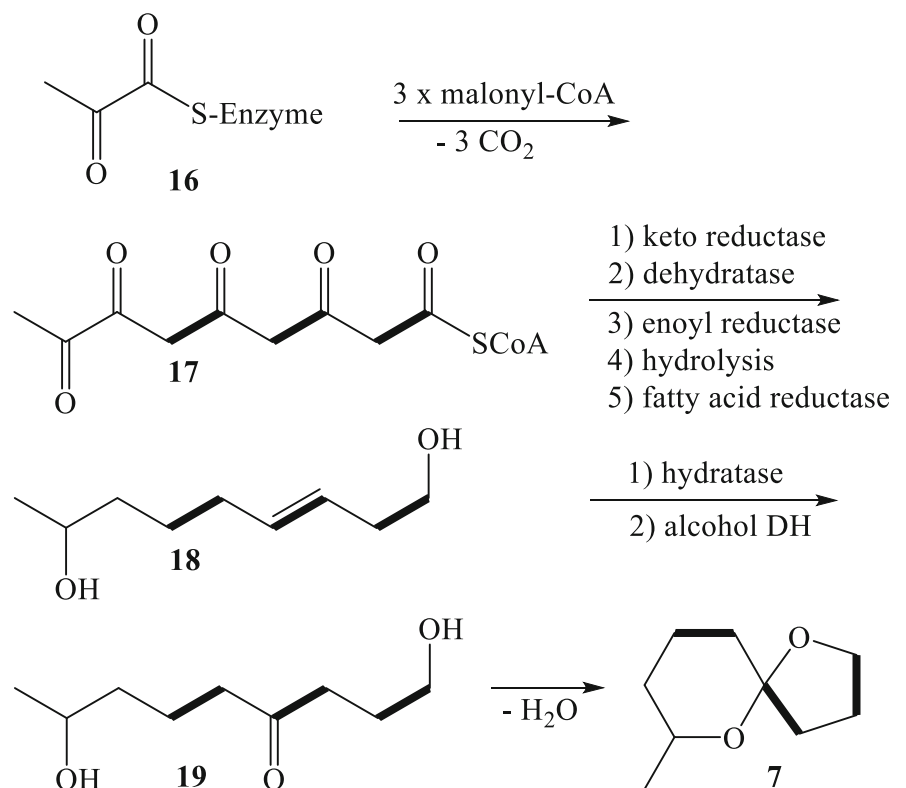
**Fig. 4** Mass spectra of chalcogran isomer 1. (A) Spectrum of unlabeled chalcogran (scan  $m/z$  from 30 to 300). (B) Spectrum of  $1\text{-}^{13}\text{C}$  labeled chalcogran (scan  $m/z$  from 30 to 300). (C) Spectrum of  $2\text{-}^{13}\text{C}$  labeled chalcogran (scan  $m/z$  from 30 to 300). (a) Spectrum of unlabeled

chalcogran (scan  $m/z$  from 125 to 135). (b) Spectrum of  $1\text{-}^{13}\text{C}$ -labeled chalcogran (scan  $m/z$  from 125 to 135). (c) Spectrum of  $2\text{-}^{13}\text{C}$ -labeled chalcogran (scan  $m/z$  from 125 to 135)

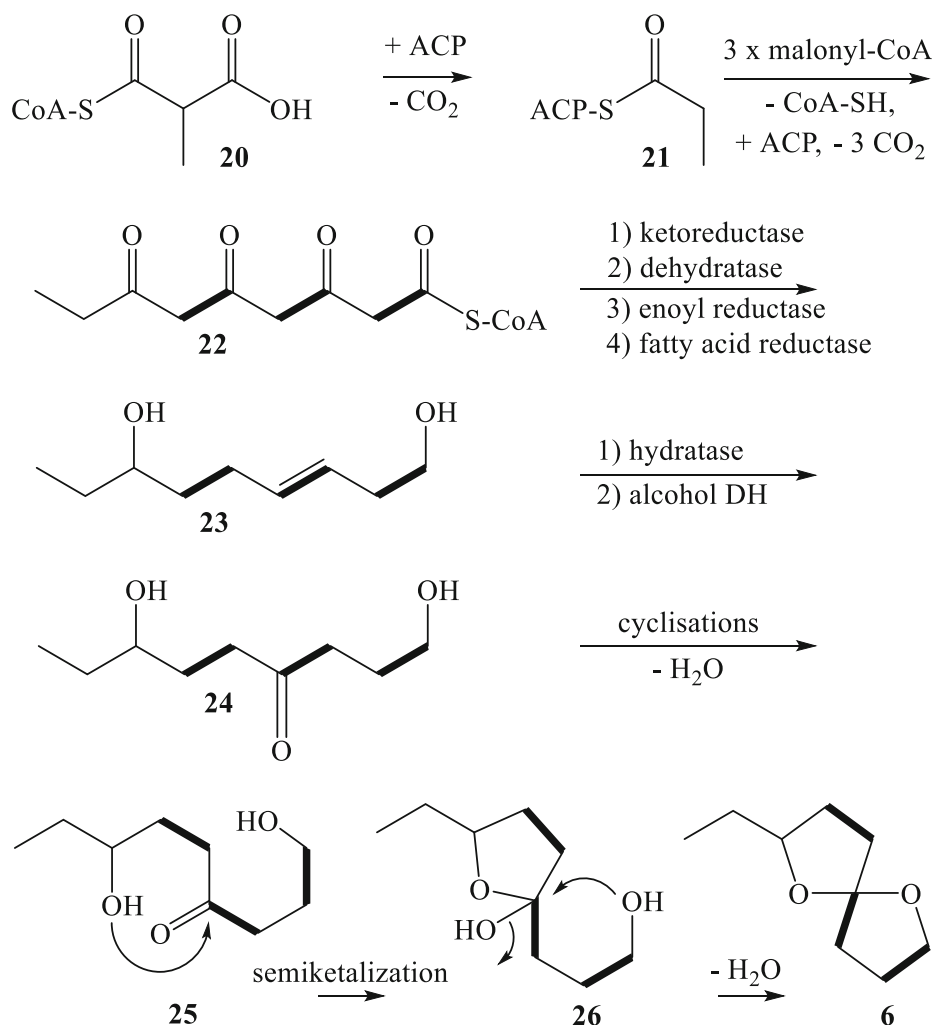


**Fig. 5** Hypothetical polyketide derived biosynthesis of 2,8-dimethyl-1,7-dioxaspiro-[5.5]undecane in *T. hirsuta* (labeled acetate extender units in bold)

**Fig. 6** Hypothetical polyketide derived biosynthesis of conophthorin in *T. hirsuta* (labeled acetate extender units to respective starter unit pyruvate in bold)



**Fig. 7** Hypothetical polyketide derived biosynthesis of chalcogran in *T. hirsuta* (labeled acetate extender units to the propanoyl starter unit in bold)



three labeled acetates are supposed to be integrated into the chalcogran molecule. Accordingly, the isotopic pattern of the EI-spectrum (Fig. 4) suggested the incorporation of at least three labeled acetate units. A fourth label, as observed in low incidence after supplementation with  $2\text{-}^{13}\text{C}$ , would require formation of the starter unit out of  $2\text{-}^{13}\text{C}$  acetate. The polyketide pathway of chalcogran biosynthesis is substantiated by the shown incorporation of at least three labeled acetates. As no intermediate of this pathway was detected in the linoleic acid-supplemented culture of *T. hirsuta*, it is most likely that the formation of the three spiroketals was catalyzed by a type I polyketide synthase. These are large multifunctional, multimodular enzymes containing all catalytic domains required for polyketide assembly and  $\beta$ -keto group processing. An antiSMASH search ([fungismash.secondarymetabolites.org/#!/start](http://fungismash.secondarymetabolites.org/#!/start)) resulted in one putative type I PKS cluster for *T. hirsuta*, only. This strongly supports the hypothesis that the spiroketals were the product of a type I

polyketide synthase. Type I polyketide synthases usually consist of the following multiple catalytic domains: starter acyltransferase (SAT), ketosynthase (KS), acyltransferase (AT), product template (PT), tandem acyl carrier proteins (ACPs), and Claisen cyclase (CLC) domains. After the respective number of Claisen condensations and reductive processing, the backbone of the polyketide is released from the enzyme (Ray and Moore 2016). To form spiroketals, the released dihydroxyketone precursors must then undergo intramolecular acetalization (Fig. 7). This reaction showed a slight preference for the (*E*)-diastereomer (20% de), which suggests a chemical cyclisation.

## Conclusions

Spiroketals of various origins have been known for a long time (Bartelt 1996). Depending on the organism, their biogenesis may



follow different pathways. For chalcogran, three different pathways have been proposed: Besides fatty acid degradation and the formation along the polyketide pathway using acetyl-/malonyl-CoA as building blocks (Booth et al. 2009; Francke et al. 1995), pathways using solely propanoyl-CoA were suggested (Vanderwel and Oehlschlaeger 2014). This study has shown that the polyketide pathway is used in the case of *T. hirsuta*. More than one spiroketal was biosynthesized from different starter units, resulting in different chain lengths and stages of polyketo-chain processing. Obviously, there is a basal formation of chalcogran and conophthorin in *T. hirsuta*, which can be boosted by flooding the acetyl-CoA pool, e.g., with linoleic acid. This overflow can also result in the formation of further spiroketals such as 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, which is the result of simple condensation repetitions of type I polyketide synthases and does not need any further specific chain processing steps. Subsequent studies are intended to clarify if short-chain polyketals are final products of a distinguished biosynthetic route or incidentally released intermediates in the synthesis of larger polyketides of the fungal metabolism.

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## Declarations

**Consent for publication** All authors agreed to publish the following work in the journal of mycological progress.

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