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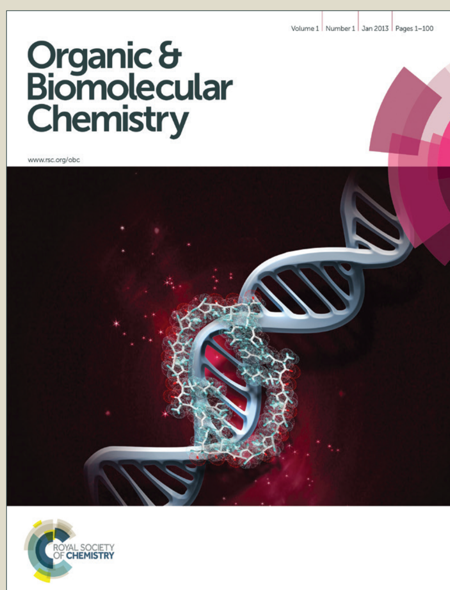

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ARTICLE

Phenalenones: Insight into the biosynthesis of polyketides from the marine alga-derived fungus *Coniothyrium cereale*

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The marine alga-derived fungus *Coniothyrium cereale* is a prolific producer of phenalenones. These polyketides were shown to possess antimicrobial effects and inhibitory activity towards the protease human leucocyte elastase (HLE). The current study focused on the biosynthesis of eight different structural types of phenalenones, comprising the natural products rousselianone A´ (**1**), coniosclerodin (**3**), cereolactam (**12**), cereoaldomine (**15**), and trypethelone (**16**). Solid agar cultures of *C. cereale* were used to follow up the incorporation of [1-^{13}C] labeled acetate into these metabolites. Taking the respective mechanisms of polyketide metabolism into account, the labeling pattern was interpreted, thus providing a hypothesis for the biosynthetic formation of the phenalenones. The polyketide skeleton of the phenanthrene-based compound cereolactam is proposed to be formed through degradation of a heptaketide by loss of two carbon atoms.

Introduction

Phenalenones are a group of natural products that are encountered in fungi and higher plants.[1] Their basic structure is a phenalene nucleus which is oxidized to a series of phenalenone derivatives. Phenylphenalenones of higher plants are recognized as phytoalexins which confer resistance towards pathogens, e.g., anigorufone in *Musa* spp.[2] The biosynthesis of these compounds in fungi and plants is completely different, in that plants use aromatic precursors from the shikimic acid pathway and malonyl CoA as building blocks, whereas in fungi these metabolites are as a whole of polyketide origin, i.e., derive merely from acetyl and malonyl units (Fig. 1).[3]

An intriguing feature of the fungal phenalenones is that they seem to undergo some oxidative degradation, yielding carbon skeleta with a reduced number of rings (Fig. S11, Supplementary information). The range of such compounds produced by the here investigated *Coniothyrium cereale* includes rousselianone A´ (**1**), lamellicolic anhydride (**2**), coniosclerodin (**3**), *Z*-coniosclerodinol (**4**), *E*-coniosclerodinol (**5**), (-)-sclerodin (**6**), (*S*,*S*)-sclerodinol (**7**), conioscleroderolide (**8**), (-)-scleroderolide (**9**), coniosclerodione (**10**), (-)-sclerodione (**11**), (-)-cereolactam (**12**), coniolactone (**13**), (-)-cereolactone (**14**), (-)-cereoaldomine (**15**), (-)-trypethelone (**16**), conioamide (**17**) and cereoanhydride (**18**) (Fig. 2).[4,5,6]

We assumed that most of these compounds (**1**-**18**) originate from a common joint heptaketide precursor, undergoing oxidative cleavage reactions (Fig. 3, hypothesis 1). Alternatively some of the compounds (**12, 13, 15,** and **16**) may be hexaketides with methyl groups derived from *S*-adenosyl-methionine (SAM) added via methyltransferases (Fig. 4, hypothesis 2). Using stable isotope-labeled precursors the origin of the carbon skeleta of the natural products was investigated. The labeling pattern of the produced polyketides was analysed by nuclear magnetic resonance spectroscopy, as well as mass spectrometric techniques (LCMS, HRMS and UPLC-HRMS). Thus, after solid phase cultivation with [1-^{13}C]-labeled acetate, sufficient amounts of these metabolites were isolated for such measurements. Taking the respective mechanisms of polyketide metabolism into account, the labeling pattern was interpreted.

Results

Time scale experiment

C. cereale produces a vast array of phenalenone derivatives (Fig. 2).[3,5,6] For the biosynthetic studies it was necessary to assess at which time point in cultivation the targeted molecules were produced in major amounts. Thus, to analyse the production of *C. cereale* metabolites, cultures harvested at different time points were assayed using LC-MS. Results of these experiments clearly indicated that the compounds coniosclerodin (**3**), (-)-sclerodine (**6**), conioscleroderolide (**8**), (-)-scleroderolide (**9**), coniosclerodione (**10**) and (-)-sclerodione (**11**) were produced from day 5 onwards, whereas rousselianone A´ (**1**) and (-)-cereolactone (**14**) were detected starting from day 7. Coniolactone (**13**) appeared at day 11 and

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(-)-trypethelone (**16**) was detected after day 13. (-)-Cereolactam (**12**) and (-)-cereoaldomine (**15**) were observed only after day 19 and 21, respectively (Table S5, Supplementary information). Since these natural products were all still present at day 30, it was concluded that the ideal time for harvesting the cultures was around 28 to 30 days, since the slightly longer cultivation elevated the amount of biomass.

Labeling experiments using solid cultures

In order to address the biosynthesis of the most unusual target compounds (**1**, **3**, **12** and **14**-**16**; Fig. 2), *C. cereal*e was cultivated with [1-^{13}C]-labeled acetate on solid culture, as most of the compounds were not produced in a liquid culture system.

To date, isotope labeling using solid cultures was hardly done, due to experimental difficulties. Indeed, precursor molecules are usually much better incorporated when fed in liquid media.[7,8] Thus, we first had to develop a methodology to optimize label uptake in solid agar-based media. A first experiment was performed by using solid Czapek dox broth medium that contained labeled acetate (0.1%) right from the beginning. This agar medium was then inoculated with a liquid pre-culture of the fungus *C. cereale*. After 30 days of cultivation, no enrichment of ^{13}C could be detected in the secondary metabolites as judged from LC-MS measurements.

Subsequently, a series of experiments revealed that several factors are important for the successful uptake of labeled precursors in solid cultures, e.g., the concentration of the labeled acetate solution, which was found to ideally be 3.0 g L^{-1}. Above all, however, the repeated periodic feeding of labeled acetate at appropriate time points is important and [1-^{13}C] labeled sodium acetate solution was supplied periodically (after days 4, 9 and 14) onto the top of the growing fungal culture. After 28 days of cultivation the culture was extracted. LC-MS analyses showed the presence of compounds **1**, **3**, **5**, **6**, **9**, **12** and **14**-**16** in the extract. The respective isotope patterns in the MS spectra indicated the incorporation of the ^{13}C label into all of these compounds. Finally, individual compounds were isolated according to Scheme S1 (Supplementary information).

The compound rousselianone A´ (**1**) is the acetone adduct of the natural product rousselianone A and represents a phenalenone with a trialicyclic ring. Compound **1** has a molecular formula of $C_{22}H_{22}O_7$, and displayed a molecular ion peak at *m/z* 399.2 $[M+H]^+$. The LC-MS spectrum clearly showed the incorporation of [1-^{13}C] labeled acetate into the core structure of compound **1**, i.e., isotopic peaks for $[M+H+1]^+$ at *m/z* 400.2, $[M+H+2]^+$ at *m/z* 401.3, $[M+H+3]^+$ at *m/z* 402.4, $[M+H+4]^+$ at *m/z* 403.5, $[M+H+5]^+$ at *m/z* 404.5, $[M+H+6]^+$ at *m/z* 405.7 and $[M+H+7]^+$ at *m/z* 406.6 (Fig. S1.3, Supplementary information).

The ^{13}C NMR spectrum (Table 1) disclosed seven resonances for ^{13}C enriched carbons, i.e., C-2, C-4, C-5, C-7, C-9, C-11 and C-14 resonating at δ_C 170.03, 138.27, 199.46, 201.45, 167.23, 150.21 and 166.67, respectively. Two more resonances were observed in the ^{13}C NMR spectrum for the ^{13}C enriched carbons C-16 (O-CH_2) at δ_C 67.27 and the quaternary carbon C-17 at δ_C 140.07, both being part of the prenyl chain in compound **1** (Fig. S9, Supplementary information). The labeling of specific carbon atoms clearly pointed out, that the basic structure of rousselianone A´ (**1**) is the product of a polyketide synthase incorporating seven acetate units into this still fully intact phenalenone nucleus (Fig. 5).

Compounds **2** – **7**, all represent the same structural type, i.e., type II (Fig. S11, Supplementary information), and only the labeling of compound **3** was analysed in detail. Coniosclerodin (**3**) with a molecular formula of $C_{18}H_{16}O_6$ (molecular ion peak at *m/z* 329.4 $[M+H]^+$) clearly showed a cluster of isotopic peaks in the LC-MS spectrum, indicating the successful incorporation of at least seven [1-^{13}C]-labeled acetates into the core structure of compound **3** (Fig. S2.3, Supplementary information). The ^{13}C NMR spectrum (Table 1; Fig. S2.2, Supplementary information) displayed nine resonances for ^{13}C enriched carbons, which were assigned to the respective carbon atoms by comparison with the ^{13}C NMR resonances of the unlabeled molecule.[4] Two of the ^{13}C enriched carbons, i.e., C-16 (O-CH_2) at δ_C 66.58 and quaternary carbon C-17 at δ_C 140.13 are part of the prenyl side chain of compound **3**. On the basis of these ^{13}C enriched carbons and our putative biosynthetic pathway (Fig. 3), we provide evidence for the formation of the polyketide part of compound **3**. Clearly seven acetate units are incorporated into the molecule, however, of the seven acetates one carbon was cleaved off (loss of carbon C-6).

The nitrogen containing metabolite (-)-cereolactam (**12**) was the next metabolite isolated from the labeled extract. It displayed a molecular formula of $C_{17}H_{17}NO_4$. UPLC-HRMS analysis showed eight isotopic peaks (Fig. S5.3, Table S2, Supplementary information) and the ^{13}C NMR spectrum (Table 1) disclosed eight resonances for ^{13}C enriched carbons, i.e. C-2, C-4, C-7, C-9, C-11, C-14, C-16 and C17. The labeling pattern is consistent with that for compound **3**, and clearly proves that carbon C-7 is acetate-derived. From this result it can be concluded that in the case of **12** the phenalenone skeleton is even more degraded as compared to **3**, i.e., of the originally present seven acetate units (14 carbons, e.g. in **1**), in **12** two carbon atoms (C-5 and C-6) are removed.

(-)-Cereolactone (**14**) with a molecular formula of $C_{17}H_{17}NO_4$ (molecular ion peak at *m/z* 301.0 $[M+H]^+$) was also obtained in pure form and its LCESIMS spectrum indicated isotopic peaks, which revealed the successful incorporation of [1-^{13}C]-labeled acetate into the compound **14** (Fig. S6.3, Supplementary information). The ^{13}C NMR spectrum (Table 1) disclosed eight resonances for ^{13}C enriched carbons (Fig. S6.2, Supplementary information), which showed that six acetate units are incorporated into the polyketide part of this molecule (whereby the last unit has lost one carbon atom, i.e. C-6) and two acetate units into the terpene unit.

The compound (-)-cereoaldomine (**15**) with a molecular formula of $C_{16}H_{15}NO_5$ was only produced in minute amounts and could not be isolated in quantities allowing NMR assessment. The HR-MS spectrum, however, showed signals indicating the incorporation of seven acetate units (Figure

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S7.1, Table S3, Supplementary information). We calculated the theoretical *m/z* values for the expected isotopic molecular ions of compound **15** and compared them with the experimental data obtained from the HR-MS measurements. The results of these calculations showed only minor deviations (<0.0010) to the experimental data. The biggest deviation was found for the $[M+H+7]^+$ isotopic molecular ion, i.e., 0.0014. It is thus suggested, that the polyketide part of **15** is labeled with five acetate units and two further ones, as seen for the other metabolites, are present in the terpene unit.

A tricyclic compound with the same carbon framework as **15**, and the molecular formula $C_{16}H_{16}O_4$ is (-)-trypethelone (**16**). The MS spectrum of the labeled compound indicated the presence of seven isotopic peaks (Figure S8.3, Table S4, Supplementary information). The ^{13}C NMR spectrum (Table 1) disclosed seven resonances for ^{13}C enriched carbons. Altogether, these data confirmed the incorporation of five acetate units in the polyketide skeleton and two into the terpenoid moiety of (-)-trypethelone (**16**).

Biosynthetic studies on (-)-sclerodin (**6**) and (-)-scleroderolide (**9**) have already been reported.[6] Spectroscopic analyses including LCESIMS and NMR techniques of compounds **6** and **9**, isolated during the current study, confirmed that they are composed of seven and six acetate units, respectively.

Discussion

The fungus *C. cereale* produces a wide range of phenalenone derivatives[5,6] with unique structural modifications (Fig. 1), which may be divided into eight structural types (Fig. S11, Supplementary information). In our former studies, these compounds were found to have potent bioactivities. Thus, compounds **3**, **6**, **8**, **12** and **15** showed significant inhibition of the protease human leukocyte elastase (HLE) with IC_{50} values of 7.2, 10.9, 13.3, 9.3 and 3.0 μM, respectively.[4,5] Sclerodione (**11**) and trypethelone (**16**) were found to be cytotoxic against mouse fibroblast cells with IC_{50} values of 6.4 and 7.5 μM, respectively.[4,5] Compound **16** exhibited considerable activity against *Mycobacterium phlei*, *Staphylococcus aureus* and *Escherichia coli*,[5] while compounds **8** and **9** displayed antimicrobial activity towards *Staphylococcus aureus* SG 511.[4]

The current study addresses the biosynthesis of these compounds. Solid agar cultures of *C. cereale* were used to follow up the incorporation of ^{13}C labeled precursors into the respective metabolites. Our study is one of the few that describes a procedure for labeling studies in agar cultures. According to our knowledge the only others being reported are the biosynthesis of zearalenone[6] and quinolactacin A, the latter being a potent inhibitor of tumor necrosis factor (TNF) production.[8]

Some biosynthetic studies have been reported for the fungal phenalenones such as deoxyherqueinone[9] and atrovenetin[10] (Fig. S10, Supplementary information) both isolated from *Penicillium herquei*. The scleroderris canker causing fungus *Gremmeniella abietina* is producing many phenalenones, i.e., sclerodine, scleroderolide, sclerodione,[11] which are similar (**6**, **9**, **11**) to the *C. cereale* metabolites. Biosynthetic studies of these metabolites (Fig. S10, Supplementary information) were performed by Ayer et al.[12] who found that sclerodine, scleroderolide, and sclerodione, were heptaketides.

A special feature of phenalenone derivatives from *C. cereale* is, that the basic tricyclic 3-hydroxy-phenalen-1-one skeleton, as found in rousselianone A, seems to be oxidatively degraded to yield metabolites with less rings and carbon atoms. As it is quite usual in fungal secondary metabolism that polyketides are substituted with methyl groups derived from *S*-adenosyl-methionine (SAM), some of the carbon atoms in *C. cereale* metabolites could originate from such an action of methyl transferases, e.g., carbon C-7 in cereolactam (**12**). This gives rise to two hypothesis: i) compounds (**1**-**18**) originate from a common joint heptaketide precursor undergoing oxidative cleavage reactions (Fig. 3, hypothesis 1), or ii) some of the compounds (**12, 13, 15,** and **16**) may be hexaketides with methyl groups added via methyltransferases (Fig. 4, hypothesis 2).

Using [1-^{13}C]-labeled acetate the origin of the carbon skeleta of these compounds were analysed, taking the respective mechanisms of polyketide metabolism into consideration. Rousselianone A´ (**1**), still having the complete carbon skeleton of phenalenones, was found to incorporate, as expected, seven acetate units (Fig. 5). The phenalenone derivative coniosclerodin (**3**) has one carbon atom, i.e., C-6, less than the heptaketide rousselianone A. Since carbons C-5 and C-7 are clearly ^{13}C labeled, it must be concluded that C-6 is lost by oxidation. Lamellicolic anhydride (**2**) with the same basic framework as **3**, was also observed in our cultures but could not be isolated in pure form. However, since compounds **2** - **7** only differ concerning the substitution of the skeleton, it can be deduced that their basic phenalenone nucleus is formed identically.

We also detected coniosclerodione (**10**) by LC-MS, which would be the expected intermediate between **1** and **3**. The naphthalene derivative **14** was found to have six acetate units incorporated, thus, it may be a hexaketide. However, if seen in the context with the other *C. cereale* metabolites it is most likely a heptaketide, whose carbon skeleton is produced by oxidative loss of C-7 from an intermediate with the basic structure of **3**. In each of the napthalenes **15** and **16**, five ^{13}C labeled carbons are found for the polyketide part of the molecules. Compound **15** was only produced in a small amount and could not be isolated for NMR analysis. We deduced the labeling pattern of compound **15** on the basis of the isotopic ions observed in HR-MS analysis. This deduction also coincided with that for (-)-trypethelone (**16**). Thus, compound **16** could be the precursor of compound **15**, after hydroxylation at C-8, transamination at the centre C-3 and oxidation of CH_3-12 to an aldehyde group (Fig. 5). Compounds **15** and **16** cannot be pentaketides, since C-3 has to be acetate derived. It has to be concluded that they are hexa- or heptaketides. Again, following the logic of the picture represented by all fungal metabolites discussed here, it may be

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assumed, that their formation involves the putative intermediate shown in Fig. 5, from which after loss of C-7 the framework for **15** and **16** originates. Alternatively this may happen via an intermediate with the basic framework of **14**.

In terms of biosynthesis, compound **12** is the most interesting one of this series. Most indicative is the ^{13}C labeling of C-7, excluding the origin of this carbon from SAM. This also necessitates a complex post-PKS machinery in the fungus, since the formation of such a branched polyketide can only occur with a heptaketide as the starter. Following the polyketide logic, even a hexaketide would not be possible as the starter unit. We thus conclude that the skeleton of compound **12** (cereolactam) is produced through oxidative loss of C-5 of a skeleton as present in **3** and lamellicolic anhydride (**2**), followed by transamination at C-3 and formation of a lactam ring.

Labeling of the prenyl moiety is consistent with the mevalonate pathway (Fig. S9; Supplementary information). According to this biosynthetic process, we observe labeling of two carbons in the prenyl moiety, each of which derived from 1-^{13}C of acetate. In compounds **6**, **9**, **12** and **14**-**16** the prenyl chain cyclized to a furanoid ring system via Claisen rearrangement (Fig. 5), as observed for atrovenetin.[13]

Conclusions

It was demonstrated that the fungal phenalenones in C. cereale are polyketides, most probably all originating from a heptaketide chain,[10,14] which cyclizes first into a tricyclic phenalenone ring system (Fig. 5). The latter is then modified by post-PKS reactions including prenylation, loss of carbons, transamination, anhydride formation, cyclisation to lactone and lactam rings. As Fig. 3 shows, this fungus produces even more related structural types (e.g. **17**, **18**) whose biosynthesis could not be addressed here because of the low amounts produced. Alltogether, this diversity proves the amazing metabolic capabilities of this fungal strain. Further insights into the biosynthesis of these compounds is currently being gained by genetic studies.

Experimental

General Procedures. All NMR spectra were measured on Bruker Avance 300 DPX and Bruker Avance 500 DRX spectrometers, respectively. Spectra were calibrated to residual solvent signals with references at $\delta_{H/C}$ 7.26/77.0 (chloroform-d_1), 2.04/29.8 (acetone-d_6) and 3.35/49.0 (methanol-d_4). All solvents of commercial grade were distilled prior to use. Silica gel 60 (Fluka, 0.040-0.063 mm/ 230-400 mesh) and Polygoprep (Macherey-Nagel, 60-50 C_{18}) were used for vacuum liquid chromatography (VLC). HPLC was performed using a Waters system, controlled by a Waters millennium software consisting of a 717 plus autosampler, a 600 E pump in combination with a 600 controller with in-line degasser and a 996 photodiode array detector, and a Merck-Hitachi system, equipped with an L-6200A pump, an L-4500 A photodiode array detector, a D-6000A interface with D-7000 HSM software. HPLC columns were from Waters (XTerra, 250 mm × 4.6 mm, 5μM) and Agilent (Zorbax Eclipse, 150 mm × 4.6 mm, 5 μM). LCESIMS measurements were conducted with an API 2000, Triple Quadrupole, LCMSMS, Applied Biosystems/MDS Sciex with ESI source. HRESIMS was measured on a Bruker Daltonik micrOTOF-Q time-of-flight mass spectrometer with ESI source. UPLC-HRMS spectra were recorded on Thermo Scientific Qexactive with HESI source (column: Phenomenex Kinetex C_{18}, 150 mm x 4.6 mm, 2.6 μM. 100 Å).

Cultivation. The marine-derived fungus *Coniothyrium cereale* was isolated from the algae *Enteromorpha* sp. (collected near Fehmarn, Baltic Sea). The fungus was isolated from the host tissues using a single colony isolation method.[15] The algae sample was rinsed thrice with sterile water, followed by sterilisation with 70% ethanol for 15 s and afterwards was again rinsed with sterile artificial seawater (ASW). Subsequently, the sterilized sample was aseptically cut into small pieces and transferred on agar plates containing the culture medium (agar 15 g L^{-1}, ASW 800 mL L^{-1}, glucose 1 g L^{-1}, peptone from soymeal 0.5 g L^{-1}, yeast extract 0.1 g L^{-1}, benzyl penicillin 250 mg L^{-1} and streptomycin sulfate 250 mg L^{-1}). The fungus growing out of the host tissues, was separated on biomalt medium (biomalt 20 g L^{-1}, agar 10 g L^{-1}, ASW 800 mL L^{-1}) until the culture was pure. The fungal strain was identified by Dr. C. Decock from the Belgian coordinated collections of microorganism of the Catholic University of Louvain (BCCM/MUCL), Belgium. A specimen (912K13) is deposited at the Institute for Pharmaceutical Biology, University of Bonn under culture collection no. 401.

For time scale experiment, 1 L Czapek medium was prepared, using Czapek dox broth (35 g L^{-1}) and agar (15 g L^{-1}). The fungal medium was transferred in 13 Petri Dishes, 30 mL in each. The fungus culture was harvested and extracted with ethylacetate (EtOAc) (20 mL for each Petri Dish), with following timespan: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 30 days. The crude extracts were monitored by LCMS and respective metabolites **1**-**18** were detected at specific retention times (Table S5, Supplementary information). The LCMS measurements were performed with following conditions: C_{18} column (Macherey-Nagel Nucleodur 100, 125 mm × 2 mm, 5 μM; flow 0.25 mL) with gradient elution from MeOH/H_2O (10/90) to 100 % MeOH in 20 min, followed by 100 % MeOH for 10 min (2 mmol NH_4OAc buffer). The results are summarized in Table S5 (Supplementary information).

For labeling studies, 1.5 L solid Czapek medium was prepared with Czapek dox broth (35 g L^{-1}), agar (15 g L^{-1}) and [1-^{13}C] labeled sodium acetate (3.0 g L^{-1}). 2.5 mL (0.3%) aqueous solution of sodium acetate was added periodically (after days 4, 9 and 14) on the growing fungus culture, during 30 days of cultivation.

Extraction and isolation. After 30 days of growth, the fungal medium was homogenized by using an Ultra-Turrax apparatus, and then was extracted with 2 L EtOAc, yielded 160 mg crude extract.

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The fungal extract was fractionated by reversed phase vacuum liquid chromatography (C_{18} VLC, Macherey-Nagel Polygoprep 60-50) using a gradient solvent system. Starting from $MeOH/H_2O$ (50/50) to 100% MeOH 11 fractions were collected. VLC fraction 2 (15.8 mg) was separated by HPLC (column: Waters XTerra C_{18}, 250 mm × 4.6 mm, 5 μM; mobile phase: acetonitrile (ACN)/H_2O (70/30); flow rate: 1 mL min^{-1}) into four sub-fractions. Sub-fraction 2.2 was further purified by HPLC (column Agilent Zorbax Eclipse C_{18}, 150 mm × 4.6 mm, 5 μM) using an isocratic elution with ACN/H_2O (70/30) with a flow rate of 1 mL min^{-1} to get compound **16** (5.0 mg) at retention time (t_R) 3.20 min. VLC fraction 2 (15.8 mg) was further analysed by UPLC HRMS (column: Phenomenex Kinetex C_{18}, 150 mm × 4.6 mm, 2.6 μM) using the following gradient solvent system: A. H_2O/0.1% formic acid; B. ACN/0.1% formic acid; 5 % B 0–2 min., 5–95 % B 2–14 min., 95 % B 14–17 min., 95–5 % B 17–22 min. The column oven was adjusted to 30°C. Compound **15** was detected at retention time (t_R) 11.17 min.

VLC fraction 5 (45 mg) was separated with a RP HPLC (column: Waters XTerra C_{18}, 250 mm × 4.6 mm, 5 μM; mobile phase: ACN/H_2O (70/30), flow rate: 1 mL min^{-1}) to get compound **12** (0.8 mg) at retention time (t_R) 4.87 min, compound **14** (4.5 mg) at retention time (t_R) of 5.32 min, compound **1** (4.3 mg) at retention time (t_R) 9.8 min, compound **3** (6.0 mg) at retention time (t_R) 10.0 min and compound **6** (4.0 mg) at retention time (t_R) 10.9 min, respectively. Fraction 5.5 (3.0 mg) was further purified by HPLC (Agilent Zorbax Eclipse C_{18} column, 150 mm × 4.6 mm, 5 μM) using an isocratic elution with ACN/H_2O (70/30) with a flow rate of 1 mL min^{-1} to get compound **5** (0.5 mg) at retention time (t_R) 7.5 min. Fraction 5.8 (4.8 mg) was further purified by HPLC (column Agilent Zorbax Eclipse C_{18}, 150 mm × 4.6 mm, 5 μM) using an isocratic elution with ACN/H_2O (70/30) with a flow rate of 1 mL min^{-1} to get compound **9** (2.0 mg) at retention time (t_R) 10.7 min.

rousselianone A´ (1). yellowish colour (4.3 mg); labeled ^{13}C NMR data (see Table 1); LCESIMS: *m/z* 399.2 $[M+H]^+$; for isotopic peaks (see Fig. S1.3, Supplementary information).

coniosclerodin (3): yellowish white powder (6.0 mg); labeled ^{13}C NMR data (see Table 1); LCESIMS: *m/z* 329.4 $[M+H]^+$; for isotopic peaks (see Fig. S2.3, Supplementary information).

(-)-sclerodin (6): yellowish white powder (4.0 mg); labeled ^{13}C NMR data (see Table 1); LCESIMS: *m/z* 329.6 $[M+H]^+$; for isotopic peaks (see Fig. S3.3, Supplementary information).

(-)-scleroderolide (9): yellowish powder (2.0 mg); labeled ^{13}C NMR data (see Table 1); LCESIMS: *m/z* 329.2 $[M+H]^+$; for isotopic peaks (see Fig. S4.3, Supplementary information).

(-)-cereolactam (12): light yellow powder (0.8 mg); labeled ^{13}C NMR data (see Table 1); HRMS: *m/z* 300.1236 $[M+H]^+$, requires 300.1236; for isotopic peaks (see Fig. S5.3, Supplementary information).

(-)-cereolactone (14): yellowish white powder (4.5 mg); labeled ^{13}C NMR data (see Table 1); LCESIMS: *m/z* 301.0 $[M+H]^+$; for isotopic peaks (see Fig. S6.3, Supplementary information).

(-)-cereoaldomine (15): UPLC HRMS: *m/z* 302.1029 $[M+H]^+$, requires 302.1028; for isotopic peaks (see Fig. 7.1, Supplementary information).

(-)-trypethelone (16): purple powder (5.0 mg); labeled ^{13}C NMR data (see Table 1); HRMS *m/z* 273.1127 $[M+H]^+$, requires 273.1127; for isotopic peaks (see Fig. S8.3, Supplementary information).

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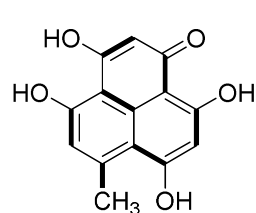
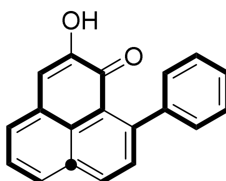
Table 1 ^{13}C NMR spectroscopic data for ^{13}C enriched compounds **1**, **3**, **6**, **9**, **12**, **14** and **16**

Pos.	Comp. 1[b] δ_C, mult.[d]	Comp. 3[a] δ_C, mult.[d]	Comp. 6[a] δ_C, mult.[d]	Com. 9[c] δ_C, mult.[d]	Comp. 12[c] δ_C, mult.[d]	Comp. 14[b] δ_C, mult.[d]	Comp. 16[b] δ_C, mult.[d]
2	170.03, C	167.98, C	164.20, C	168.12, C	151.18, C	160.72, C	176.64, C
4	138.27, C	135.09, C	135.31, C	124.13, C	131.73, C	135.62, C	135.55, C
5	199.46, C	164.72, C	164.84, C	171.90, C	-	167.17, C	-
7	201.45, C	165.15, C	165.41, C	-	165.79, C	-	-
9	167.23, C	165.22, C	165.93, C	148.44, C	138.33, C	138.38, C	162.56, C
11	150.21, C	150.70, C	149.72, C	138.06, C	143.50, C	131.65, C	142.74, C
14	166.67, C	166.68, C	166.13, C	170.99, C	157.75, C	164.35, C	175.12, C
16	67.27, CH_2	66.58, CH_2	14.51, CH_2	14.82, CH_3	14.66, CH_3	14.64, CH_3	14.92, CH_3
17	140.07, C	140.13, C	43.38, C	44.25, C	44.23, C	44.21, C	43.86, C

[a] In chloroform-*d*$_1$. [b] In acetone-*d*$_6$. [c] In methanol-d$_4$. [d] Implied multiplicities determined by DEPT.



phenalenone



anigorufone

Fig. 1 Basic structures of phenalenone derivatives of fungi and phenylphenalenones of higher plants (example anigorufone) and their proposed biosynthetic building blocks; Phenalenone: heptaketide; anigorufone: 2 x C_6C_3 + malonyl CoA (•)

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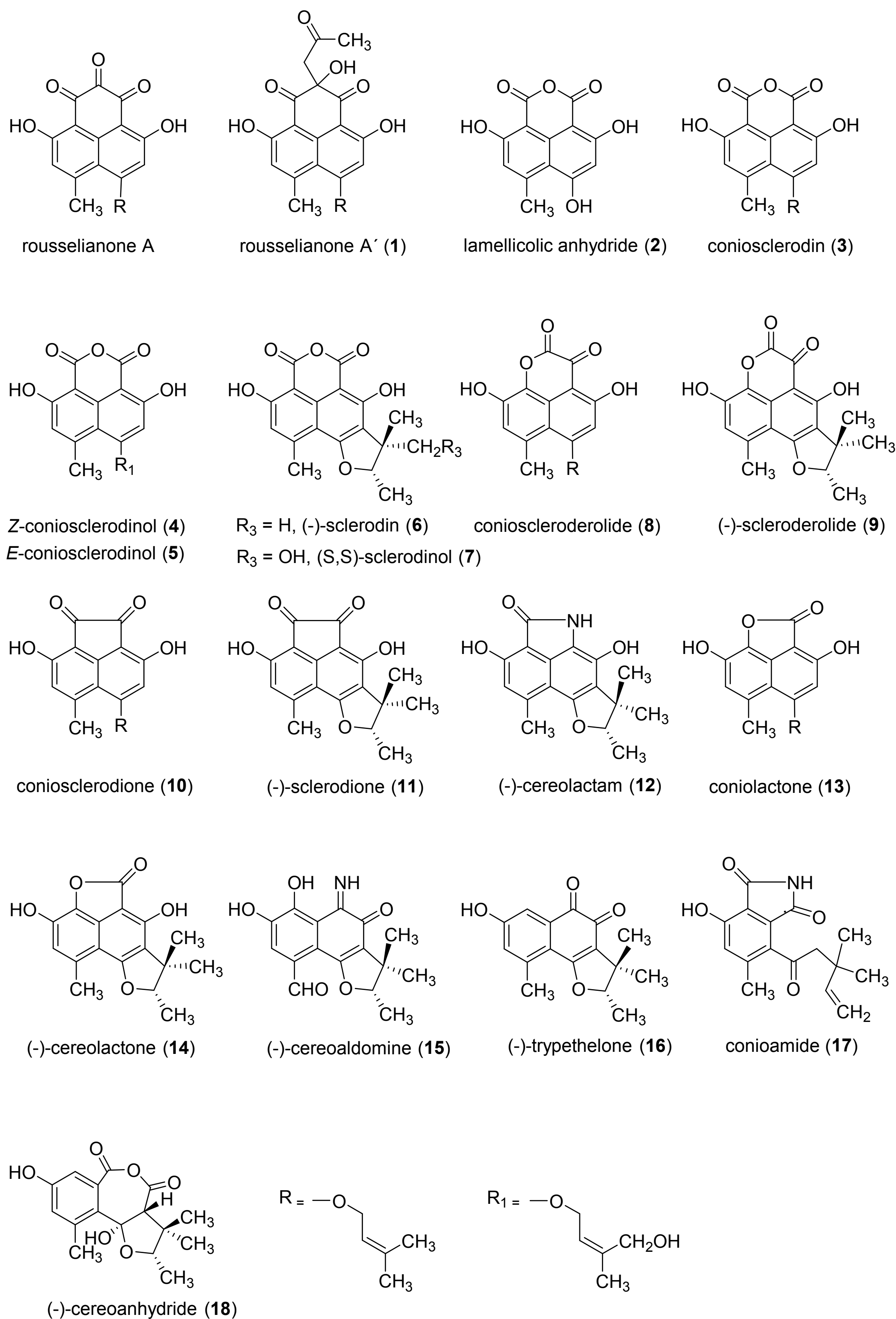

Fig. 2 Structures of polyketides (**1-18**) produced by the marine algae-derived fungus *C. cereale*. Compound **1,** rousselianone A', is an acetone adduct of rousselianone A and thus an artefact.

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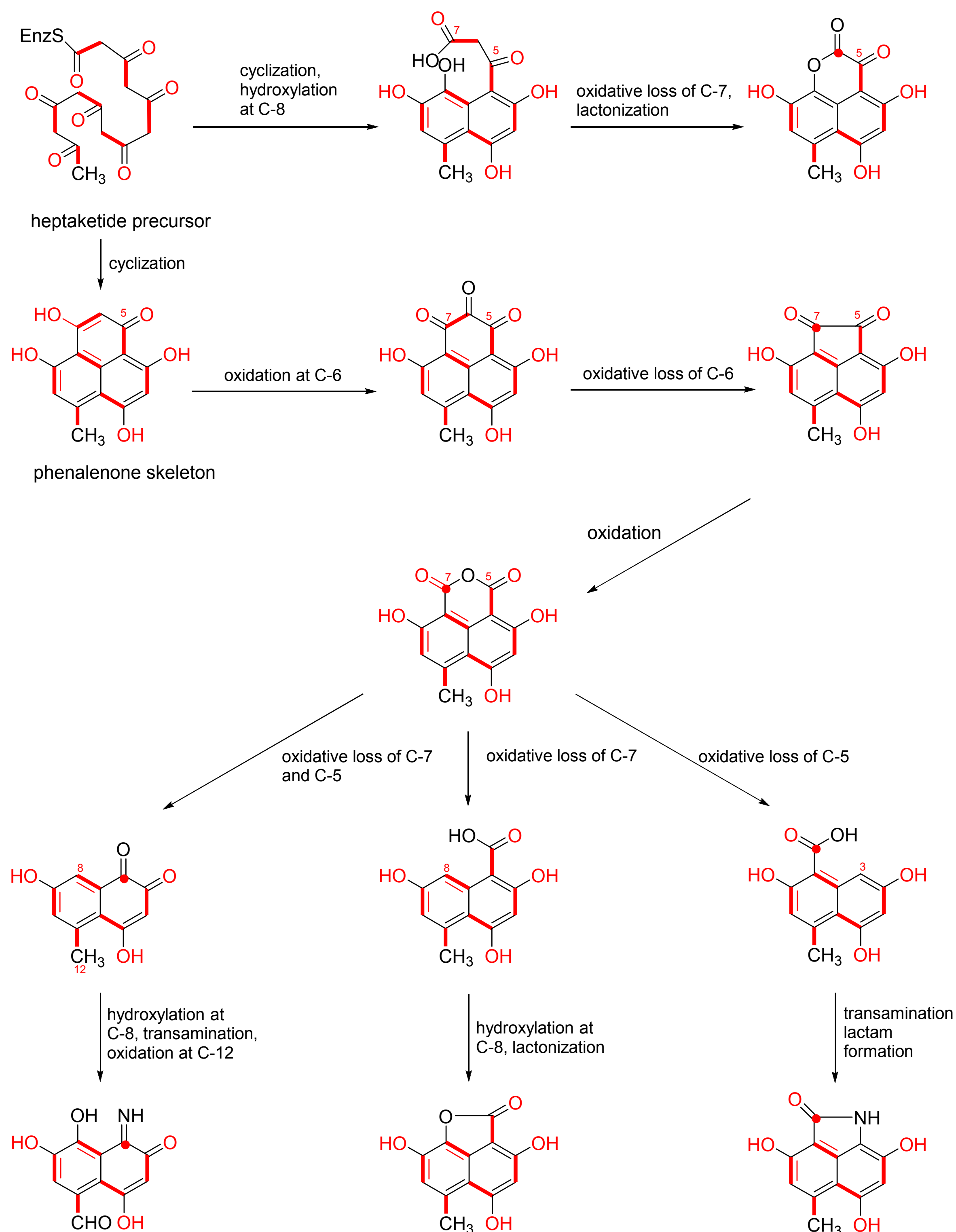

Fig. 3 Putative biosynthesis of *C. cereale* metabolites from a heptaketide precursor (**Hypothesis 1**).

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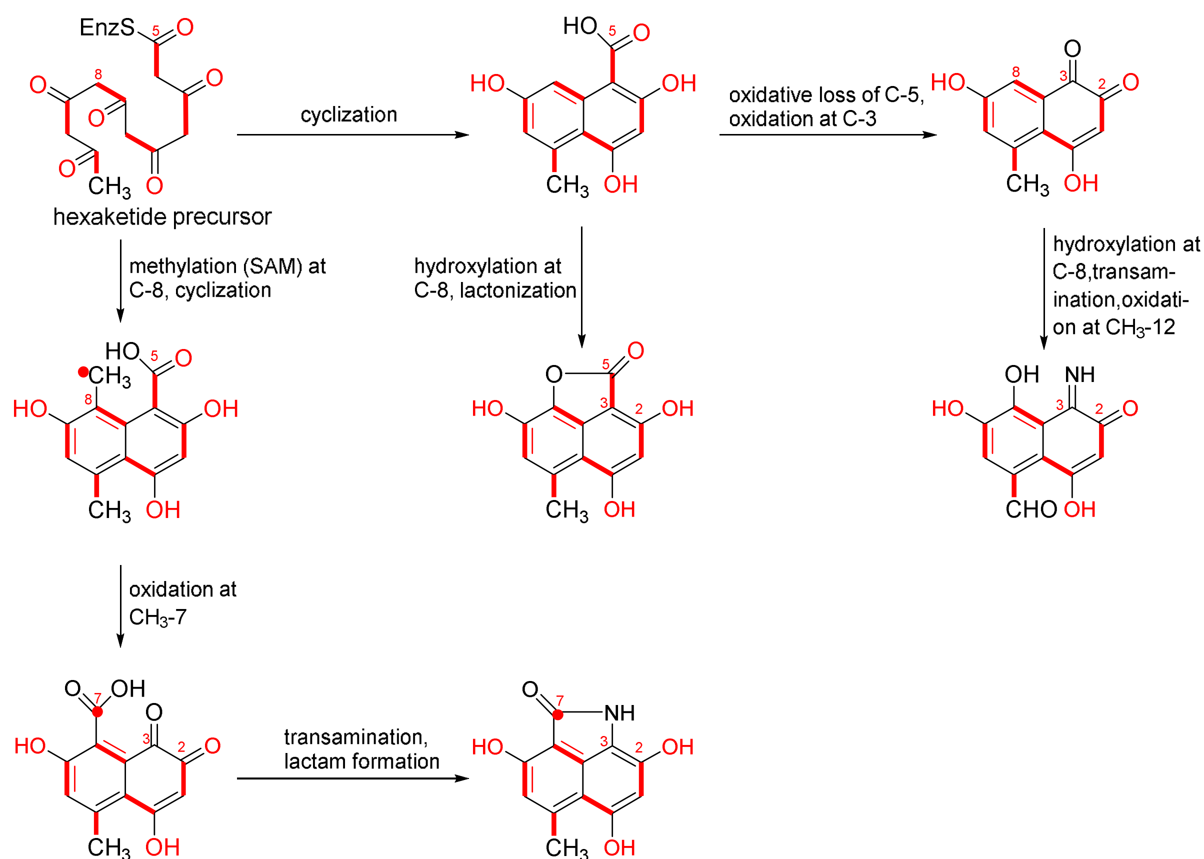

Fig. 4 Putative biosynthesis of *C. cereale* metabolites from a hexaketide precursor (**Hypothesis 2**); *S*-adenosylmethionine (SAM): (•)

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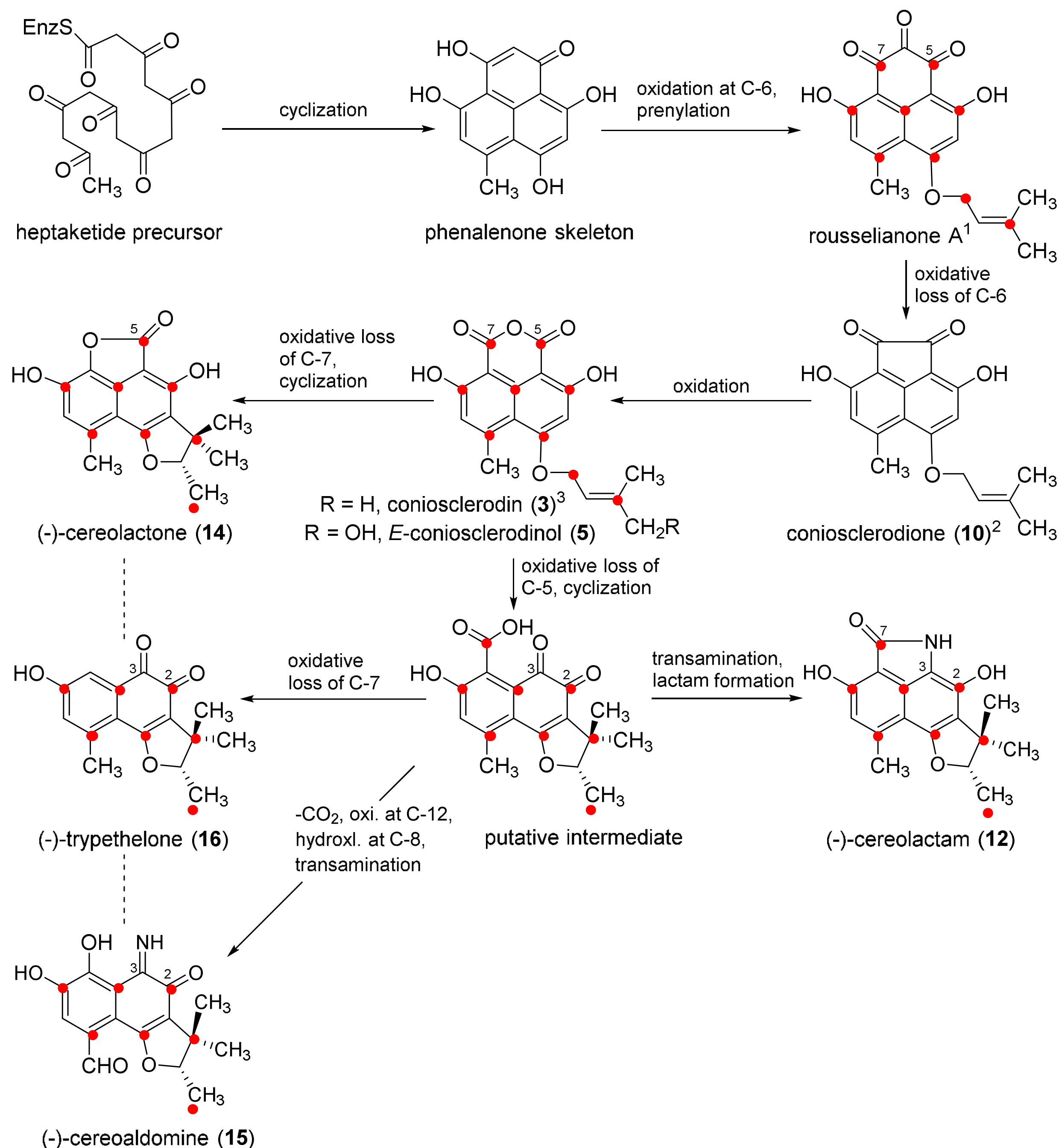

Fig. 5 Biosynthetic scheme for *Coniothyrium cereale* polyketides based on the results of [1-^{13}C] acetate feeding. The scheme covers all structural types shown in Fig. S11) apart from IV.
Dotted line = alternative paths
[1] labeling pattern as observed in rousselianone A' (**1**)
[2] obtained in non-labeled form from *C. cereale* extracts
[3] also present in non-labeled and not prenylated form, i.e. lamellicolic anhydride (**2**) in fungal extract

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