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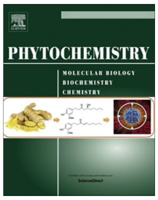

A study on the biosynthesis of hygrophorone B^{12} in the mushroom *Hygrophorus abieticola* reveals an unexpected labelling pattern in the cyclopentenone moiety

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ABSTRACT

The hitherto unknown natural formation of hygrophorones, antibacterial and antifungal cyclopentenone derivatives from mushrooms, was investigated for hygrophorone B^{12} in *Hygrophorus abieticola* Krieglst. ex Gröger & Bresinsky by feeding experiments in the field using ^{13}C labelled samples of D-glucose and sodium acetate. The incorporation of ^{13}C isotopes was extensively studied using 1D and 2D NMR spectroscopy as well as ESI-HRMS analyses. In the experiment with [U-$^{13}C_6$]-glucose, six different $^{13}C_2$ labelled isotopomers were observed in the 2D INADEQUATE spectrum due to incorporation of [1,2-$^{13}C_2$]-acetyl-CoA. This labelling pattern demonstrated that hygrophorone B^{12} is derived from a fatty acid-polyketide route instead of a 1,4-α-D-glucan derived anhydrofructose pathway. The experiment with [2-^{13}C]-acetate revealed an unexpected incorporation pattern in the cyclopentenone functionality of hygrophorone B^{12}. Four single-labelled isotopomers, in particular [1-^{13}C]-, [2-^{13}C]-, [3-^{13}C]-, and [4-^{13}C]-hygrophorone B^{12}, were detected that showed only half enrichment in comparison to the respective labelled alkyl side chain carbons. This labelling pattern indicates the formation of a symmetrical intermediate during hygrophorone B^{12} biosynthesis. Based on these observations, a biogenetic route *via* a 4-oxo fatty acid and a chrysotrine B homologue is discussed.

1. Introduction

Fungal fruiting bodies of the basidiomycetous genus *Hygrophorus* (Hygrophoraceae, Agaricales) are an exceptional source for chemically diverse secondary metabolites, such as γ-butyrolactones (Gill and Steglich, 1987; Lübken et al., 2004), a dihydroazepine (Gill and Steglich, 1987), a ceramide (Qu et al., 2004), (nor-)sesquiterpenes (Otto et al., 2014), 4-oxo fatty acids (Teichert et al., 2005a; Gilardoni et al., 2006), cyclopentenediones (Gilardoni et al., 2007), and cyclopentenones (Lübken et al., 2004; Bette et al., 2015). The latter compound class, named hygrophorones A–E, represents 2-cyclopentenones with hydroxy or acetoxy substituents at C-4 and C-5, to which an odd-numbered 1′-oxidised alkyl chain (C_{11}, C_{13}, C_{15}, C_{17}) is attached (Lübken et al., 2004; Schmidts et al., 2013).

Very recently, the metabolite hygrophorone B^{12} (**1**) was isolated from fruiting bodies of *Hygrophorus abieticola* Krieglst. ex Gröger & Bresinsky and subsequently synthesised in an enantiomerically pure form, allowing for an unambiguous determination of the absolute configuration (Bette et al., 2015). Despite synthetic examinations, the *in vivo* formation of hygrophorones remains yet to be investigated. Biogenetic pathways can be studied by feeding experiments using stable-isotope labelled precursors (e.g. ^{2}H, ^{13}C, ^{15}N) in combination with an NMR-based analysis of incorporation patterns.

In theory, the biogenesis of hygrophorones, in particular hygrophorone B^{12} (**1**), might follow one of the two pathways shown in Scheme 1. Due to its structural similarity to oxidised fatty acids, **1** may be biosynthesised *via* an acetyl-CoA derived fatty acid-polyketide metabolism (pathway A). Another possible biogenesis of **1** includes the separate formation of a cyclopentenone unit followed by stereospecific alkylation at C-5 (pathway B). In this case, pentenomycin (**2**) may serve as precursor (Umino et al., 1973), which could be formed from 1,4-α-D-glucans over anhydrofructose (**3**) (Baute et al., 1988), similarly to its diastereomer epipentenomycin in the ascomycete *Peziza echinospora* (Baute et al., 1991). The latter pathway B is supported by identification in fungi with structurally very similar compounds, while pathway A is supported by our finding of fatty acids with a 4-oxo-crotonate moiety as potential intermediates (Teichert et al., 2005b).

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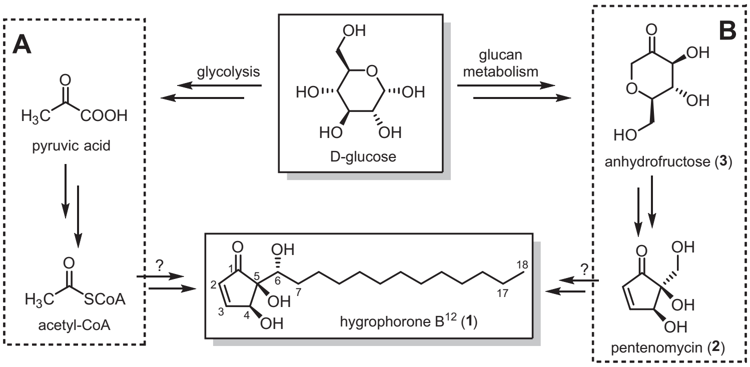

Scheme 1. Possible biogenetic pathways to hygrophorone B^{12} (**1**) starting from D-glucose. (A) Glycolysis to acetyl-CoA and subsequent fatty acid-polyketide biosynthesis; (B) 1,4-α-D-glucan metabolism to anhydrofructose (**3**) and pentenomycin (**2**) followed by a stereospecific alkylation.

The present study describes *in vivo* feeding experiments on fruiting bodies of *H. abieticola* using ^{13}C labelled samples of D-glucose and sodium acetate in order to investigate the hitherto unknown biosynthesis of hygrophorone B^{12} (**1**).

2. Results and discussion

Feeding experiments on *H. abieticola* were performed in the field allowing the mushrooms to grow under natural conditions. To guarantee an efficient incorporation of labelled precursors into **1**, young fruiting bodies were selected due to their high rate of secondary metabolic activity. For this purpose, aqueous solutions of [U-$^{13}C_6$]-glucose, [1-^{13}C]-glucose, or [2-^{13}C]-acetate were injected separately *via* a syringe into the caps and stems of fruiting bodies of *H. abieticola* and harvested seven to nine days after feeding.

To investigate the biosynthesis of hygrophorone B^{12} (**1**) with special focus on the cyclopentenone functionality, [U-$^{13}C_6$]-glucose can be used as a precursor for both possible biogenetic routes described in Scheme 1. Both pathways will consequently generate different incorporation patterns in the cyclopentenone moiety, leading to different NMR signatures (Fig. 1). Thus, a putative formation of **1** following the anhydrofructose route would result in an uniformly ^{13}C labelled ring system leading to a double doublet (dd) NMR signal due to ^{13}C–^{13}C coupling between adjacent ^{13}C nuclei. In turn, a fatty acid-polyketide biogenesis would lead to an $^{13}C_2$ isotopomeric labelling pattern in the cyclopentenone moiety of **1** arising from incorporation of [1,2-$^{13}C_2$]-acetyl-CoA at the various positions, along with biogenic unlabelled acetyl-CoA. As a result, a doublet (d) signal splitting is observed.

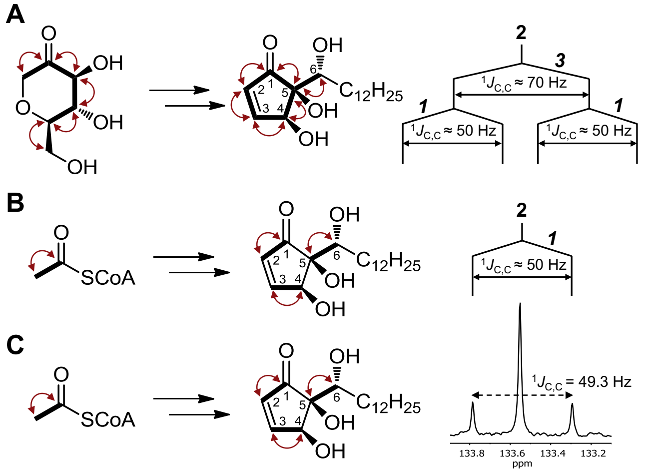

Fig. 1. Predicted and observed ^{13}C labelling patterns of the cyclopentenone moiety in **1** after incorporation of [U-$^{13}C_6$]-glucose and resulting NMR signatures exemplified for C-2 (right side). ^{13}C–^{13}C couplings are indicated by arrows and bold bonds. (A) predicted for anhydrofructose pathway; (B) predicted for acetyl-CoA derived biogenesis; (C) observed labelling pattern.

To examine these putative biogenetic pathways, each of five basidiocarps of *H. abieticola* were fed with 100 mg of [U-$^{13}C_6$]-glucose. Hygrophorone B^{12} (**1**) was isolated from the EtOAc extract of *H. abieticola* fruiting bodies by repeated sequential fractionation on silica gel SPE cartridges followed by preparative HPLC (see Section 4). Up to 6 mg of hygrophorone B^{12} (**1**) can be obtained from a single fruiting body. The 1D NMR and ESI-HRMS data of **1** were in accordance to those reported recently in the literature (Bette et al., 2015).

The proton-decoupled ^{13}C NMR spectrum of hygrophorone B^{12} (**1**) after feeding of [U-$^{13}C_6$]-glucose revealed an efficient incorporation of $^{13}C_2$ units as predicted for an acetyl-CoA based pathway (Fig. 2). All ^{13}C signals were segregated into doublets caused by $^1J_{C,C}$ coupling between neighboured ^{13}C isotopes at high intensities of approximately 52% in comparison to the global singlet signals (Table 1). Coupling carbon pairs were suggested from satellite signals with similar coupling constants. Both the cyclopentenone ring and the alkyl side chain carbon atoms of **1** were equally labelled exhibiting an average absolute ^{13}C enrichment of 0.56% per carbon atom (see Supplementary Information, S2). The incorporation of $^{13}C_2$ units was corroborated by ESI-HRMS measurements showing a significant enrichment of the [M+2] and [M+4] ion abundances (Table 2). The slightly increased intensity of the [M+1] ion, however, may be explained by indirect metabolism of glucose *via* several pathways, yielding single-labelled acetyl-CoA.

For an unambiguous confirmation of enriched carbon atom pairs in hygrophorone B^{12} (**1**) after feeding of [U-$^{13}C_6$]-glucose, a two-dimensional INADEQUATE experiment was performed. As depicted in Fig. 3, this ^{13}C homonuclear correlation experiment allowed the identification of $^1J_{C,C}$ couplings as pairs of antiphase doublet peaks along a horizontal line (Hull, 1994). The distance between these antiphase doublets reveals the coupling constant of the respective ^{13}C–^{13}C coupling. In total, six different $^{13}C_2$ labelled isotopomers, namely [1,2-$^{13}C_2$]-, [3,4-$^{13}C_2$]-, [5,6-$^{13}C_2$]-, [7,8-$^{13}C_2$]-, [15,16-$^{13}C_2$]-, and [17,18-$^{13}C_2$]-**1**, were detected (Fig. 3). As a result of signal overlap of C-9 to C-15 resonating around δ_C 30, coupling pairs for these carbons could not be assigned. Based on the observed labelling pattern of the other alkyl chain carbons, however, it is proposed that the carbons C-9 to C-15 are labelled as well. The ^{13}C NMR data including INADEQUATE correlations and $^1J_{C,C}$ coupling constants of **1** after feeding of [U-$^{13}C_6$]-glucose are summarised in Table 1. The incorporation pattern of $^{13}C_2$ units into hygrophorone B^{12} (**1**) is in agreement with a

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