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Biosynthetic experiments with tall plants under field conditions. $^{18}O_2$ incorporation into humulone from *Humulus lupulus*

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This work is dedicated to Professor Wolfgang Steglich on the occasion of his 70th birthday.

Abstract

Five segments of a large hop plant (*Humulus lupulus* var. Hallertauer Magnum) carrying several cones were enclosed in sealed glass vessels that were gassed with $^{18}O_2$. After 14 days, the segments were harvested and humulone and cohumulone were analysed by NMR spectroscopy and mass spectroscopy. The oxygen atoms in position 6 of humulone and cohumulone showed 9% ^{18}O enrichment, respectively. It follows that the C-6 hydroxy groups were introduced by oxygenase catalysis.

Keywords: *Humulus lupulus*; Cannabaceae; Biosynthesis; Bitter acid; Humulone; Cohumulone

1. Introduction

Biosynthetic studies of secondary plant metabolites can be conducted with intact plants, segments of plant tissue, plant cell cultures or plant cell extracts. These approaches can not be applied easily in cases where the product under study is exclusively or predominantly produced in specific organs of large plants under field conditions but not inside the laboratory. As an example, the bitter acid, humulone (**1**, Fig. 1), as well as its ad-derivative **2** and co-derivative **3**, are accumulated in considerable amounts in the glandular cells of hop (*Humulus lupulus*). In most hop varieties, humulone is the predominant representative of bitter acids. During the brewing process, the α-acids are isomerised into more stable and soluble iso-forms which are important for the taste as well as the stability of beer (for review, see Verzele, 1986). The humulone content is a major factor in assessing the commercial value of different hop varieties, and increasing the humulone content is a major aim in hop breeding.

Incorporation experiments using ^{14}C-labeled acetate or leucine into humulone had suggested a polyketide-type biosynthesis with isovaleryl–CoA as starter unit (Drawert and Beier, 1976a,b). Furthermore, incorporation experiments using ^{13}C labeled glucose isotopomers have shown that the biosynthesis of humulone (**1**) involves two molecules of the terpenoid precursor, dimethylallyl diphosphate (**7**), which is biosynthesised via the deoxyxylulose phosphate pathway (Goese et al., 1999). More recently, in vitro experiments showed that phloroisovalerophenone (**6**) is obtained from isovaleryl–CoA (**4**) and three molecules of malonyl–CoA (**5**) by the catalytic activity of purified phlorisovalerophenone synthase (Fig. 2) (Paniego et al., 1999). In this paper we describe in vivo incorporation experiments with molecular $^{18}O_2$ in order to elucidate the biosynthetic origin of the C-6 oxygen atoms of humulone (**1**) and cohumulone (**3**).

2. Results and discussion

In order to study the origin of the oxygen atoms in humulone (**1**) and cohumulone (**3**), we performed a field study using an intact hop plant with a height of about 3

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m. Segments of the intact plant carrying several cones were enclosed in glass vessels which were sealed with candle wax and silicon wax (Fig. 3). The vessels contained approximately 20% $^{18}O_2$ and 80% Ar. After 14 days, the cones were harvested. Humulone and cohumulone were isolated and analysed by NMR spectroscopy and mass spectrometry.

The ^{13}C-NMR signals of C-6 in humulone (**1**) and cohumulone (**3**) showed satellites (indicated by arrows in Fig. 4) with upfield shifts of 21 ppb, which are in the typical range of ^{18}O-shifts in alcohols (Risley and van Etten, 1990) (17–35 ppb). The relative intensity of the ^{18}O-shifted signals accounted for 9% of the total signal intensities. No satellites were observed in the ^{13}C-NMR spectra of the other oxygen-substituted carbon atoms (Fig. 4). Mass spectrometry showed increased $(M+2)^{+\bullet}$ ions for humulone and cohumulone, as compared to natural abundance materials, which indicated ^{18}O enrichments of approximately 10%. It follows that, in growing hop cones, the oxygen atoms at C-6 of humulone and cohumulone were derived from elementary oxygen. The incorporation indicates that the relative

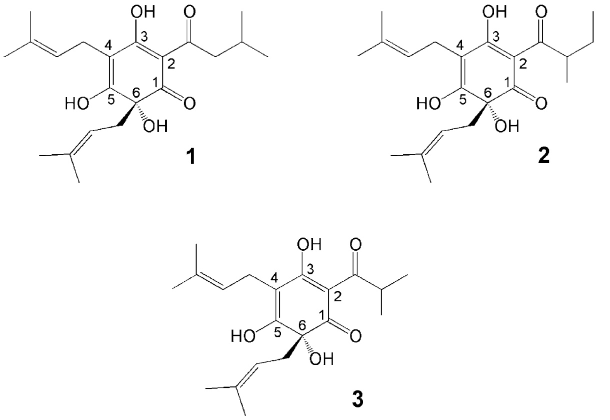

Fig. 1. Structures of humulone (**1**), adhumulone (**2**) and cohumulone (**3**).

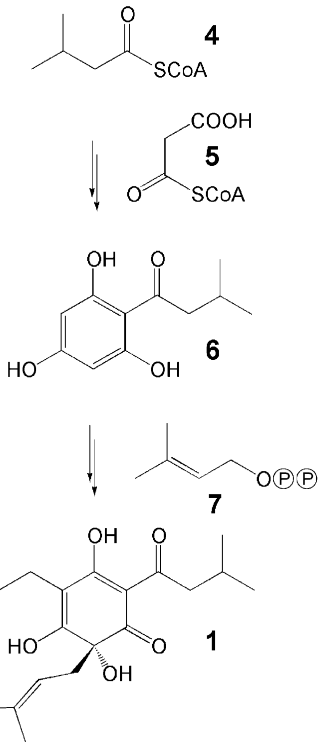

Fig. 2. Hypothetical biosynthetic pathway of humulone (**1**).

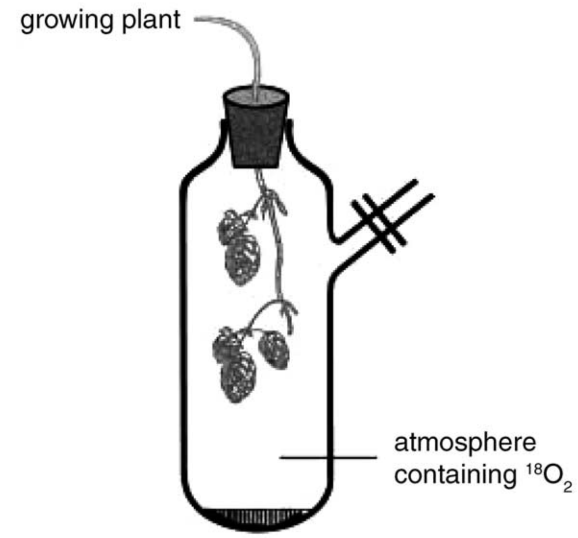

Fig. 3. Experimental setting used in this study for the in vivo incorporation of $^{18}O_2$.

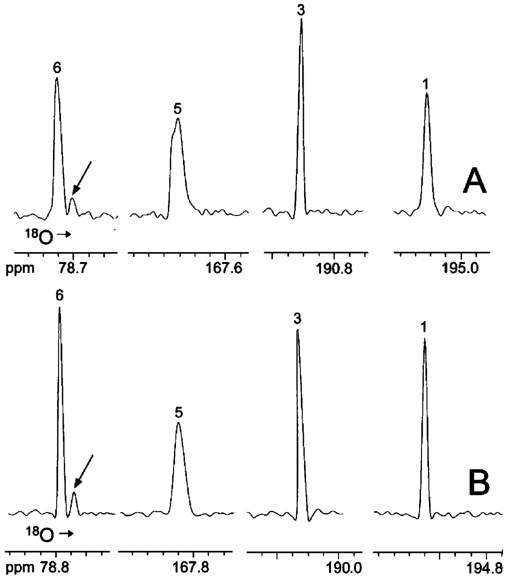

Fig. 4. ^{13}C-NMR signals of humulone (A) and cohumulone (B) from the incorporation experiment with $^{18}O_2$. Signals shifted by ^{18}O-isotope effects are indicated by arrows. The numbers on top of the signals indicate ring carbon atoms with attached oxygen atoms. The numbering schemes are given in Fig. 1.

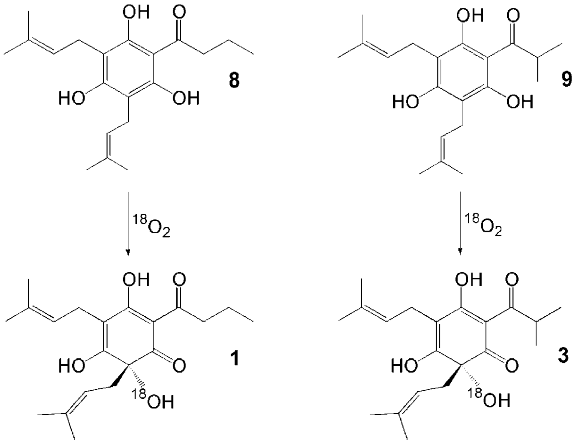

Fig. 5. Biosynthetic conversion of deoxyhumulone (**8**) and deoxycohumulone (**9**) into humulone (**1**) and cohumulone (**3**), respectively, in the presence of $^{18}O_2$.

amount of humulone or cohumulone that was biosynthesized during the feeding period was approximately 10%. Not surprisingly, about 90% of the bitter acids were already present in the hop cones at the beginning of the labeling experiment.

Our in vivo data confirm that the known intermediate **6** is converted into deoxyhumulone (**8**) which is then converted into humulone (**1**) by reaction with molecular oxygen. Similarly, the corresponding deoxycohumulone (**9**) has been transformed into cohumulone (**3**) by molecular oxygen (Fig. 5). In line with these results, it was shown earlier that hop protein extracts catalyze the conversion of deoxyhumulone (**8**) into humulone (**1**) in the presence of air (Fung et al., 1997).

The experimental approach used in the present study represents an example for the analysis of the biosynthetic origin of oxygen atoms using large, intact plants which cannot be cultured easily under laboratory conditions.

3. Experimental

3.1. Materials

$^{18}O_2$ was obtained from Cambridge Isotope Laboratories (Andover, Massachusetts).

3.2. NMR spectroscopy

Humulone or cohumulone were dissolved in 0.5 ml of $CDCl_3$, respectively. ^{13}C-NMR spectra were recorded at 126 MHz and at a temperature of 20 °C with a Bruker DRX 500 spectrometer. Prior to Fourier transformation the free induction decays were multiplied with Gaussian window functions. The signal assignments were taken from Goese et al. (1999).

3.3. Mass spectrometry

Measurements were performed with a Finnigan MAT 95 double-focusing mass spectrometer with reversed Nier-Johnson geometry. EI mass spectra were recorded at 70 eV.

3.4. Incorporation experiment with $^{18}O_2$

Five plant segments each carrying 7 cones (approximate length; 1 cm) of an intact plant (*Humulus lupulus*, L. var. Hallertauer Magnum) were sprayed with a fungicide (Paral®, Pilzfrei N) and were then enclosed in glass vessels (one segment per vessel) (Fig. 3). Each vessel contained a bag of silica gel in order to absorb excess water. The flasks were sealed with a stopper, candle wax and silicon wax (Silicon Hochleistungsfett, Wacker GmbH, Germany) and flushed with argon to replace $^{16}O_2$. The pressure in the vessels (170 ml) was then reduced to 714 mbar, and $^{18}O_2$ was added to a total pressure of 1020 mbar. The vessels were shaded with aluminium foil. The cones were harvested after two weeks.

3.5. Isolation of humulone (*1*) and cohumulone (*3*)

The plant material (approximately 1.4 g, dry weight) was extracted with three 50 ml portions of dichloromethane at 20 °C. *o*-Phenylendiamine (50 mg) was added, and the solution was filtered and evaporated to dryness under reduced pressure. The residue was dissolved in a mixture of methanol/water/formic acid (85:17:0.25) and purified by preparative HPLC (Sharpe and Ormrod, 1991) using a RP-18 column (YMC-PACK ODS-AQ, 11 µm, 40×250 mm) and an eluent containing methanol/water/formic acid (85:17:0.25). The flow rate was 70 ml min^{-1}. The retention times of cohumulone and humulone were 16 and 20 min, respectively (yields, 20 mg of each compound).

Acknowledgements

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