A Labeling Study To Elucidate the Biosynthesis of 4-(4-Hydroxyphenyl)-Butan-2-one (Raspberry Ketone) by *Nidula niveo-tomentosa*

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Submerged cells of the basidiomycete *Nidula niveo-tomentosa*, a microbial producer of 4-(4-hydroxyphenyl)-butan-2-one (p-HPB) have been found in numerous plant genera, such as *Artemisia*, *Capparis*, *Dendrobiuim*, *Hippophae*, *Larix*, *Limonium*, *Pinus*, *Prunus*, *Rheum*, *Rubus*, *Saxifraga*, *Taxus*, *Vaccinium*, *Vanilla*, and *Vitis*, and in the glands of the melon fly, *Dacus cucurbitae*, and of the North American beaver, *Castor canadensis* (4, 8, 9, 13, 17, 20, 24). Surface cultures of the basidiomycete *Nidula niveo-tomentosa* were reported as another source as early as 1980 (2).

The methyl ketone showed antibacterial activity (19) and activity as an attractant for pollinating bees (10) and fruit flies (21). In mammalian metabolism, a skin-lightening activity was caused by a selective growth inhibition on human melanotic adipocytes (15). The strongest interest in the compound, however, since its structural identification as the aglycon of rheosmin, is in its character impact function in raspberry flavor (3). As its concentration in food plants is far too low for economical isolation, efforts were made to open a biotechnological route, based on submerged culture of *N. niveo-tomentosa* (4). The natural form of this sought-after aroma compound may cost up to $10,000/kg.

Supplementation of a fungal production medium with either L-phenylalanine or L-tyrosine increased the yields of phenylbutanoid compounds significantly to levels beyond 100 mg liter$^{-1}$. However, immediate development of the bioprocess to a productivity level sufficient for industrial-scale production failed (5). It was therefore decided to elucidate the fungal pathway in more detail. This should provide sound biochemical knowledge to aid in selecting metabolic precursors for further optimization. The phenylpropanoyl precursor in plant biochemical studies was p-coumaryl coenzyme A (CoA) (1, 6, 7). Decarboxylative condensation with labeled malonyl-CoA was demonstrated for cell extracts of raspberry fruit and tissue cultures. Intracellular levels of intermediates and further metabolism of p-HPB were monitored in raspberry suspension cultures (18). The key enzyme benzalacetone synthase was enriched from ripe raspberries (7), and the same plant-specific enzyme and a cDNA encoding it were isolated from rhubarb (1).

By using the more food-compliant and convenient precursor L-phenylalanine as the phenylpropanoyl moiety, it was attempted to confirm the plant pathway to p-HPB in the high-yielding fungal metabolism of *N. niveo-tomentosa* (12). The pattern of ring-labeled L-[2H5]phenylalanine was recovered from p-HPB, from the corresponding 4-(4-hydroxyphenyl)-butan-2-ol (betuligenol and rhododrendrol), from the α,β-enzyme, and from 3-hydroxy-5-(4-hydroxyphenyl)-pentanoic acid, demonstrating the role of L-phenylalanine as a facultative precursor and also indicating a side chain-elongating capability of the fungus. When the cells were exposed to L-[1-13C]phenylalanine in concentrations up to 10 mmol liter$^{-1}$, no isotope effects occurred (12). If the fungus had used the plant pathway, the label of L-[1-13C]phenylalanine should have had to be recovered from fungal p-HPB and the related volatile metabolites; this, however, was not observed (12). The objective of the present study was to resolve this apparent contradiction and to attract attention to a novel method of analyzing stable isotope-labeled metabolites, gas chromatography-atomic emission detection (GC-AED).

**MATERIALS AND METHODS**

**Microorganisms.** The examined strains of *N. niveo-tomentosa* were from the Centraalbureau voor Schimmelcultures (CBS strain 380.60), Baum, The Netherlands, and the American Type Culture Collection (ATCC 38357), Manassas, Va. All quantitative data refer to the CBS strain. The ATCC strain gave comparable results; both strains are ex soil under pteridium.

**Chemicals.** All chemicals for culture media were purchased from Merck (Darmstadt, Germany). Raspberry ketone and acetoniure (high-performance liquid chromatography [HPLC] grade) were supplied by Aldrich (Taiirkichen, Germany). Labeled phenylalanines were from Promoechem, Wesel, Germany; labeled glucose was from Chemotrade, Düsseldorf, Germany. (+)-Diclofop-methyl (propanoic acid, 2-[4-(2,4-dichlorophenoxy)pheny], methyl ester) was...
from Riedel-de Haen, Seelze, Germany. The solvents used for extraction were of analytical grade and distilled before use.

**Cultivation.** Strains were inoculated (homogenized mycelium) into 100 ml of culture medium (20 ml for the experiments carried out with t-[13C]glucose) and grown aerobically at 24°C in 300-ml Erlenmeyer flasks on a rotary shaker (Mültitron; Infors, Bottmingen/Basel, Switzerland). The standard culture period was 14 to 16 days, after which the formation of volatile metabolites leveled off. Each day aliquots of the culture broth were centrifuged, glucose consumption and dry weight were measured according to standard protocols, and the supernatants were extracted immediately for metabolite analysis. The culture medium contained 50 g of glucose monohydrate liter⁻¹, 2 g of soy peptone liter⁻¹, 1.5 g of yeast extract liter⁻¹, 2.5 g of KH₂PO₄ liter⁻¹, 0.5 g of MgSO₄·7H₂O liter⁻¹, 73.5 mg of CaCl₂·2H₂O liter⁻¹, and 1.0 ml of trace element solution (Fe, Zn, Cu, and Mn ions) liter⁻¹ (soy peptone medium; pH 6.0).

**Metabolite extraction.** The centrifuged culture broths were extracted with pentane-diethyl ether (anisotropic mixture). The organic phases were dried over anhydrous Na₂SO₄; concentrated in a Vigreux column; and analyzed directly or after methylation, silylation, or acetylation by capillary gas-liquid chromatography with different systems, as indicated in the text.

**GC analysis.** The solvent extracts were analyzed by capillary gas-liquid chromatography with a Fisons 8000 gas chromatograph. Chromatographic conditions were as follows: splitless injection; injection port, 230°C; flame ionization detector, 270°C; carrier gas, hydrogen; flow rate, 3.8 ml min⁻¹. Chromatograms were evaluated with HP ChemStation software (Agilent Technologies, Palo Alto, Calif.).

An HP 6890 gas chromatograph with a Gerstel KAS 4 injection system, coupled with an HP G2520 atomic emission detector (GC-AED), was used for isotope analysis with the following conditions: programmed temperature vaporization, 40 to 240°C (10°C); atomic emission detector, 280°C; carrier gas, helium; inlet pressure, 20 lb/in². The GC-AED technique provides rapid and selective, in part also simultaneous, measurement of most elements of the peri-atomic array. The minimum detectable level is 1 pg of 2H/s at 655,990 nm and 10 pg of 12C and the 13C trace. A proportion of 45% of the phenylbutanoids was found to be labeled. MS data resulted in a calculated labeling proportion of about 44%. This meant that each 23rd (10 carbon atoms: 44 × 100) atom of a phenylbutanoid compound actually was a 13C atom. Higher electronic amplification was therefore necessary for equal signal height, which caused the higher signal-to-noise ratio seen in the lower trace of Fig. 1.

**RESULTS AND DISCUSSION**

**Labeling experiments with t-phenylalanines.** Suspended pellets of *N. niveo-tomentosa* tolerate exogenous t-phenylalanine in concentrations up to 10 mmol liter⁻¹ without microscopic changes or effects on growth. A previous time course study of the incorporation of ring-perdeuterated phenylalanine over 16 days did not show a pronounced isotope effect or scrambling of the added isotope label (12). The addition of 3 mmol of sterile-filtered t-[2-13C]phenylalanine liter⁻¹ resulted in an empty AED chromatogram for the 13C trace (data not shown), just like the addition of the 1-13C-isotopomer in the preceding study (12). This indicated correct subtraction of the natural 12C background but also nonincorporation of 1- and 2,13C of the phenylalanine side chain. Accordingly, no isotope labeling of phenylbutanoid compounds was found by GC-MS. 13C-labeled target compounds occurred only upon feeding of t-[3-13C]phenylalanine. Figure 1 compares 12C and 13C traces of the GC-AED: the chromatographic 13C trace almost perfectly mirrored the 12C trace, although only 1 out of the 10 carbons of the phenylbutanoid products could contain a 13C atom derived from the precursor molecule and although nonlabeled phenylalanine was present in the soy peptone used as the nitrogen source. Evaluation of the 12C/13C labeling ratios of the benzylic carbon atom of both p-HPB and betulinol by GC-AED is possible, if equal response factors of the two carbon isotopes are assumed in integrating the peak areas of the 12C and the 13C trace. A proportion of 45% of the phenylbutanoids was found to be labeled. MS data resulted in a calculated labeling proportion of about 44%. This meant that each 23rd (10 carbon atoms: 44 × 100) atom of a phenylbutanoid compound actually was a 13C atom. Higher electronic amplification was therefore necessary for equal signal height, which caused the higher signal-to-noise ratio seen in the lower trace of Fig. 1.

-p-[4,13C]HPB showed an intense molecular ion at *m/z* 165 which upon α-cleavage of the C—C bond in benzylic position yielded a ring-extended hydroxytroplium ion at *m/z* 108 (nonlabeled counterpart, *m/z* 107); other important fragment ions were HOPH[13CH₂]₂CO⁺ at *m/z* 150 and HOPH[13CH₂]₂CH⁺ at *m/z* 122. The similar mass spectrum of betulinol is distinguished by a fragment ion resulting from elimination of water at *m/z* 149 (nonlabeled counterpart, *m/z* 148) and by the propenyl fragment ion HOPH[13CH₂]₃CH⁺ at *m/z* 134 resulting from the loss of the terminal methyl group (and water).

The presence of labeled 4-(4-hydroxyphenyl)-3-buten-2-one is consistent with the general route of polypeptide synthesis: reduction of the β-keto function followed by elimination of water would yield just this intermediate. Hydrogenation of the α,β double bond concludes the sequence, delivering a saturated hydrocarbon chain.

**Formation of the side chain.** The growth of a hydrocarbon chain is brought about by a *Clausen*-type condensation reactions of activated acetate or acetate plus malonate units (11). A partial replacement of glucose in the nutrient medium by equimolar amounts of acetate, malonate, dimethyl malonate, pyruvate, l-alanine, oxalacetate, 2-oxoglutarate, hexadecanoic acid, or plant oils resulted in similar or decreased concentrations of p-HPB and betulinol during the growth cycle. As the endogenous acetyl pool appears to be replenished by glucose as the main C source of the nutrient medium, d-[1-13C]glucose was supplied together with 3 mmol of nonlabeled t-phenylalanine liter⁻¹. This should yield 2-13C-labeled acetyl-CoA and, upon carboxylation, 2-13C-labeled malonyl-CoA. If the benzyl starter unit was elongated by twofold condensation of malonyl-
CoA units, C1- and C3-labeled phenylbutanoid compounds had to be received. Indeed, a GC-AED chromatogram very similar to that in Fig. 1 was obtained after 12 days of cultivation in the presence of D-[1-13C]glucose. From the intensity of the fragment ions a proportion of 24.4% of labeled p-HPB was derived. The mass spectrum of p-HPB showed additional isotope fragments at m/z 165 and, with clearly lesser intensity, m/z 166 (nonlabeled molecular ion M+ at m/z 164) resulting from monolabeled (1- or 3- position) and from double-labeled (1- and 3- position) metabolites, respectively. With the label in positions 1 and 3 of the butyl side chain, cleavage of the COC bond in benzylic position yielded again the (here non-labeled) hydroxytropylium ion at m/z 107 and the phenyl cation at m/z 77. An α cleavage next to the keto group led to HOPhCH2[13CH2]CO+ at m/z 150, HOPhCH2[13CH2]CO+ at m/z 122, and [13CH3]CO+ at m/z 44 (nonlabeled m/z 43). The mass spectral data further indicate that scrambling of the glucose label did not occur: a labeled carbon in the 2- position would have been detected in the mass spectrum of the ketone by m/z 45 ([13CO-13CH3]) and in betuligenol by m/z 47 ([13CHOH-13CH3]), but both ions were completely absent. As the fourth carbon of the chain is obviously derived from phenylalanine, the glucose label cannot occur there. The elongation of a benzyl moiety by addition of a three-carbon unit, an alternative route that could be envisaged, for example, through a reduction of the terminal carboxylate of added malonate, is also excluded by the observed labeling patterns. These patterns were obtained throughout the entire cultivation period.

Other labeled metabolites. According to GC-AED and GC-MS analyses the 13C label was also contained in the α,β-enone (peak 3 in Fig. 1), presumably the immediate progenitor of betuligenol (see above discussion), and in two metabolites with M+ 196 and isotope fragments at m/z 197 and 198. Evaluation of the mass spectra pointed to the presence of 4-(4-hydroxy-3-methoxyphenyl)-butan-2-ol (zingerol) and another methoxy derivative of betuligenol (compounds 4 and 5 in Fig. 1). The concentrations were too low for GC-preparative enrichment and nuclear magnetic resonance spectrometry. Microchemical characterization gave the following results: methylation did not alter the structure of both compounds, silylation and acetylation indicated the presence of two hydroxy groups, and the acetylated derivatives yielded fragment ions typical of one aromatic (M+ 42) and aliphatic hydroxy (M+ 60) groups (Fig. 2). Hydroxylation of aromatic rings and subsequent methylation of the phenolic function are common in basidiomycete biochemistry (22). These compounds may contribute to the pool of monomers used by the fungus for the assembly of higher-molecular-weight metabolites (2).

Inhibition of acetyl-CoA-carboxylase. Several arylphenoxyp propaneoates, such as diclofop-methyl, inhibit the carboxylation of acetyl-CoA and can thus be expected to decrease the formation of phenylbutanoids by N. niveo-tomentosa. In the presence of 3 mmol of L-phenylalanine liter−1 and 0.5 mmol of diclofop-methyl liter−1, growth was not retarded; only after more than 2 weeks of cultivation was premature entrance into stationary phase, compared to that of a control culture, ob-

FIG. 1. GC-AED chromatogram of an extract from N. niveo-tomentosa upon addition of 3 mmol of L-[3-13C]phenylalanine. Compound designations: 1, p-HPB; 2, 4-(4-hydroxyphenyl)-butan-2-ol; 3, 4-(4-hydroxyphenyl)-3-buten-2-one; 4 and 5, structure isomers of hydroxymethoxyphenyl-butan-2-ol. 12C was detected at 177.491 nm, and 13C was detected at 176.934 nm.
A concentration of 0.1 mmol of inhibitor liter\(^{-1}\) only slightly altered the spectrum of products, but at a concentration of 1 mmol liter\(^{-1}\) the inhibitor blocked the formation of the phenylbutanoid compounds in favor of the formation of mainly benzyl compounds (Table 1). This inhibition could not be overcome by the addition of up to 20 mmol of dimethyl malonate. Even in the absence of an inhibitor, millimolar supplements of sodium malonate or malonyl dimethyl ester did not increase the concentration of phenylbutanoid compounds. This failure to cross-access a metabolic pathway is a common observation when multienzyme complexes are involved.

Further indications for the suggested pathway. Benzoates or 4-HO-benzoates, respectively, could serve as an exogenous benzyl starter for further chain elongation by the fungus. Supplementation in millimolar amounts did, however, not increase the formation of phenylbutanoid compounds but retarded growth. In view of the worldwide use of these compounds as antifungal food additives, no other result was expected. Likewise, no precursor activity was found for 3-HO-3-phenylpropanoic acid and its 3-keto analogue, when supplied as ethyl esters in millimolar amounts. These hypothetical intermediates will occur as activated, enzyme-bound metabolites, saturating the following enzyme on site. Hence, exogenous substrate will be ignored because of the multienzyme complex nature of the catalysis involved. The lack of measurable concentrations of free intermediates other than those shown in Fig. 1 is in line with this view.

N-Benzoyl glycine (hippuric acid) was able to replace L-phenylalanine as a facultative precursor of \(\text{p-HPB}\) (Table 2). Growth curves indicated little acute toxicity (data not shown). While feeding with 10 mmol liter\(^{-1}\) reduced the yields of phenylbutanoid compounds, indicating an initial adverse effect, 1 mmol liter\(^{-1}\) resulted in product formation almost as high as that by 3 mmol of L-phenylalanine liter\(^{-1}\) (molar conversion rate of about 52%). The ratio of phenylbutanoid alcohol to ketone was around 91/9 throughout these experiments. It is supposed that the superior precursor efficiency of N-benzoyl glycine originated from the metabolic use of both the benzyl and the glycine moiety of the amide. N-Benzoyl glycine hydrolases occur widely in microorganisms (16). However, high hydrolase activity would be suicidal; 1 mmol of free benzoic acid already was exerting toxic effects as judged by growth inhibition. Thus, the observed efficiency of N-benzoyl glycine might rather be attributed to its function as a chemical cache of a benzyl moiety under feedback regulation by the fungus. Alternatively, the monocharged amide may pass membrane barriers more easily than phenylalanine might.

The studies feeding labeled and nonlabeled precursor substrates suggest the fungal provision of a benzyl starter which is then side chain elongated by a twofold condensation to form compounds possessing a phenylbutanoid skeleton, with D-glucose acting as an efficient malonyl donor (Fig. 3). As was presumed previously (12), this pathway is obviously different from the one well established previously for raspberry (6, 7, 18) and rhubarb (1), where phenylbutanoids were formed via one-step decarboxylative condensation of 4-coumaryl-CoA with malonyl-CoA. While benzalacetone synthase from rhubarb was cloned and sequenced, the respective fungal enzyme was not reported yet. The reductive decarboxylation of a phenylpentanoic moiety with accumulation of phenylbutanoids is a sin-

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### TABLE 1. Compounds identified in gas chromatograms of extracts from nutrient media of *N. niveo-tomentosa* upon supplementation with diclofop-methyl, an acetyl-CoA-carboxylase inhibitor

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound concn (mg liter(^{-1})) at inhibitor concn (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>p-HPB</td>
<td>0.7</td>
</tr>
<tr>
<td>4-(4-Hydroxyphenyl)-butan-2-ol</td>
<td>8.7</td>
</tr>
<tr>
<td>4-(4-Hydroxyphenyl)-butan-2-one</td>
<td>0.2</td>
</tr>
<tr>
<td>Phenylacetic acid</td>
<td>ND</td>
</tr>
<tr>
<td>2-Phenylethanol</td>
<td>ND</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>ND</td>
</tr>
<tr>
<td>4-Methoxybenzoic acid</td>
<td>ND</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>ND</td>
</tr>
<tr>
<td>Phenylmethanol</td>
<td>ND</td>
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\(^a\) ND, not detected.
gular step in microbial biochemistry, and no microorganism besides \textit{N. niveo-tomentosa} is known to be capable of this reaction. The formation of phenylbutanoid compounds may be explained by a premature release of intermediates from a polyketide synthase complex incapable of regularly handling longer-chain intermediates. This deficiency, however, becomes easily observable only in the presence of a large supply of exogenous benzyl precursor leading to pathway overflow: in the absence of added precursor, the total concentration of phenylbutanoids in a submerged culture in standard nutrient medium without complex ingredients is only in the range of 2 to 3 mg/liter (12 days of cultivation).

Preliminary attempts to reproduce the bioconversion by using cell extracts prepared according to a standard protocol (cell disintegration with a glass ball mill, 0°C; phosphate buffer pH 7.5), or according to the method in reference 6, have so far been unsuccessful but will be the subject of future experiments. The use of other specifically labeled precursors, such as L-3-D-phenylalanine, could provide additional evidence for the suggested metabolic pathway. The role of N-benzoyl glycine and of N-benzoyl glycine hydrolases will be investigated by monitoring the time course of liberation of benzoic acid in the nutrient medium. Another objective will be to locate experimental conditions for shifting the formation of 4-(4-hydroxyphenyl)-butan-2-ol more toward the actual biotechnological target, raspberry ketone.

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\section*{REFERENCES}


