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Enantiomeric ratios of 2-methylbutanoic acid and its methyl ester: Elucidation of novel biogenetic pathways towards (*R*)-methyl 2-methylbutanoate in a beverage fermented with shiitake

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2-Methylbutanol (PubChem CID: 8723)
2-Methylbutanal (PubChem CID: 7284)
2-Methylbutanoic acid (PubChem CID: 8314)
(*S*)-2-Methylbutanoic acid (PubChem CID: 448893)
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ABSTRACT

Up to 35% of (*R*)-methyl 2-methylbutanoate (M2MB) was observed in a beverage fermented with shiitake. As M2MB naturally occurs typically in high excesses of the (*S*)-enantiomer, the origin of the (*R*)-ester was elucidated by stable isotope labeled precursor-feeding studies. (*R*)-2-Methylbutanoic acid was identified as the main precursor in the substrate wort. Trace amounts of (*R*)-M2MB were produced by transformation of unsaturated secondary metabolites (tiglic aldehyde and tiglic acid) derived from L-isoleucine. Surprisingly, shiitake esterified (*R*)-2-methylbutanoic acid faster to (*R*)-M2MB than the corresponding (*S*)-enantiomer. Concurrently, spontaneous non-enantioselective degradation of M2MB occurred in shiitake. This explains diverse enantiomeric ratios of M2MB and different enantiomeric ratios of 2-methylbutanoic acid and M2MB in the beverage. As the odor threshold values of (*R*)-and (*S*)-M2MB differ significantly, these findings are of high relevance for the overall flavor of the fermented beverage and elucidate the discrepancy of enantiomeric ratios of 2-alkyl-branched acids and esters reported in nature.

1. Introduction

Esters imparting fruity odor impressions occur in small amounts (generally less than 100 mg/L) widely in fermented beverages (*e.g.*, beer and wine) (Hu, Jin, Mei, Li, & Tao, 2018; Soles, Ough, & Kunkee, 1982; Sumby, Grbin, & Jiranek, 2010; Verstrepen et al., 2003). These esters are highly important for the flavor profile of the drinks. Due to their low odor thresholds and synergistic effects, minor changes in their concentrations might have significant effects on the flavor of the beverages (Hammond, 1993). Recently, a novel non-alcoholic beverage fermented by shiitake was developed using unhopped wort as a substrate (Zhang, Fraatz, Horlamus, Quitmann, & Zorn, 2014). An ester, methyl 2-methylbutanoate (M2MB) biosynthesized by shiitake, was identified as the most important flavor compound of the beverage. A strong correlation between the concentration of M2MB and the perceived typical fruitiness of the beverage was confirmed (Zhang, Hartung, Fraatz, & Zorn, 2015). These findings imply that the elucidation of the ester formation by shiitake is essential for achieving the best possible product. To the best of our knowledge, there is little information available on the biosynthesis pathways of M2MB, although

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the compound has been found in fruits (*e.g.*, apples (Rowan, Lane, Allen, Fielder, & Hunt, 1996), cactus pears (Weckerle et al., 2001), muskmelons (Schieberle, Ofner, & Grosch, 1990), strawberries (Pérez, Olías, Luaces, & Sanz, 2002), pineapples (Spanier et al., 1998)), herbs (dill – *Anethum graveolens*) (Blank & Grosch, 1991), and mushrooms (*Polyporus tenuiculus*) (Omarini, Henning, Ringuelet, Zygadlo, & Albertó, 2010). In contrast, the biogenetic routes to the ethyl ester of 2-methylbutanoic acid, ethyl 2-methylbutanoate (E2MB), have been well documented for apples and yeasts using deuterium-labeled precursors (Díaz-Maroto, Schneider, & Baumes, 2005; Rowan et al., 1996). Starting from L-isoleucine (2*S*, 3*S*) the exclusive presence of (*S*)-configured 2-methylbutanoic acid and E2MB is plausible. However, the inconsistency of enantiomeric ratios of 2-methylbutanoic acid and E2MB in other foodstuffs has been revealed in many studies (Matheis, Granvogl, & Schieberle, 2016; Rettinger et al., 1991). This may indicate that complex biogenetic pathways exist.

A biosynthetic pathway towards M2MB starting from L-isoleucine *via* 2-methylbutanoic acid as an intermediate was confirmed in our previous study (Zhang, Hartung, et al., 2015). Because M2MB is a chiral odorant, its optical purity, odor quality, and knowledge about concurrent pathways are of utmost importance, to gain control over the ester's formation during the production and storage of the beverage. Therefore, the enantiomeric ratios of M2MB were analyzed systematically by means of a multi-dimensional gas chromatograph-mass spectrometer (MDGC-MS) system. Meanwhile, the biogenetic pathways of M2MB formation, especially for its (*R*)-enantiomer, have been elucidated in detail. Finally, the discrepancy of enantiomeric ratios between 2-methylbutanoic acid and M2MB was investigated.

2. Materials and methods

2.1. Materials and chemicals

Shiitake (*Lentinula edodes*) was obtained from the *Centraalbureau voor Schimmelcultures* (CBS, Utrecht, Netherlands). Wort (Koelsch type, 13° Plato) was provided by the THM University of Applied Sciences (Giessen, Germany) (Zhang et al., 2014).

L-Isoleucine (U-^{13}C) (98%) was purchased from Euriso-Top GmbH (Saarbrücken, Germany). (*S*)-Methyl 2-methylbutanoate (98%) was obtained from Wonda Science (Jiangsu, China). (*S*)-2-Methylbutanoic acid (98%) and (*R*)-2-methylbutanoic acid (98%) were bought from VWR (Darmstadt, Germany) and Biozol Diagnostica Vertrieb GmbH (Eching, Germany), respectively. Other reference compounds were purchased from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Steinheim, Germany), TCI Deutschland (Eschborn, Germany), Th. Geyer (Hamburg, Germany), and VWR (Darmstadt, Germany). For gas chromatography, helium 5.0 (Praxair, Düsseldorf, Germany) and nitrogen 5.0 (Linde, Munich, Germany) were used.

2.2. Synthesis of (R)-methyl 2-methylbutanoate (M2MB)

(*R*)-2-Methylbutanoic acid (28.9 mg) was added to boron trifluoride dissolved in methanol (20%, 3 mL) and incubated for 5 min at 80 °C. After cooling to room temperature, hexane (3 mL) was added and the reaction mixture was incubated for 1 min at 80 °C. The mixture was washed with a saturated aqueous solution of sodium chloride (5 mL), and the organic phase was dried over anhydrous sodium sulfate overnight.

2.3. Production of the fermented beverage with shiitake

The pre-culture of shiitake was prepared as described previously (Zhang et al., 2014; Zhang, 2015). The mycelium of 10 mL pre-culture broth was precipitated by means of centrifugation (2150*g*, 10 min, 20 °C) and washed three times with sterile water. The fungal pellets were re-suspended in 10 mL sterilized wort, and the suspension was transferred into an Erlenmeyer flask (250 mL) containing 100 mL sterilized wort. The fermentation was carried out at 24 °C under aerobic conditions on a rotary shaker (150 rpm, shaking diameter 25 mm).

2.4. Headspace solid-phase microextraction-gas chromatography system equipped with a tandem mass spectrometer and an olfactory detection port (HS-SPME-GC–MS/MS-O)

For HS-SPME, a CAR/PDMS (Carboxen™/polydimethylsiloxane, 75 µm, 1 cm) (Supelco, Steinheim, Germany) fiber in combination with an MPS 2XL multipurpose sampler (GERSTEL, Mülheim an der Ruhr, Germany) was used. The same procedure of sample preparation and parameters of HS-SPME and GC–MS/MS-O as described in our pervious study were applied to analyze all 2-methyl-1-butanol derivatives apart from tiglic acid (Zhang, Hartung, et al., 2015; Zhang, 2015). In order to trap tiglic acid by HS-SPME, the samples' pH was adjusted to 3 with HCl and a polar PA fiber (polyacrylate, 85 µm, 1 cm) (Supelco) was chosen. The concentrations of all target compounds were estimated *via* the internal standard (IS) method: 50 µL of IS (8.88 mg/L, thymol in aqueous solution with ethanol) were supplemented to 10 mL sample. The corresponding response factors were calculated for each analyte individually (Zhang, Fraatz, et al., 2015; Zhang, 2015).

2.5. HS-SPME-MDGC-MS

For HS-SPME, a CAR/PDMS/DVB fiber (Carboxen™/divinylbenzene/polydimethylsiloxane, 50/30 µm, 1 cm) in a manual fiber holder (Supelco) was used. Sodium chloride (3 g) was added to the sample (10 mL). The samples were magnetically stirred at 250 rpm in a water bath adjusted to 60 °C for 20 min, followed by headspace extraction at the same temperature for 40 min. Afterwards, the analytes were directly desorbed in the split/splitless inlet of the MDGC-MS system at 250 °C for 1 min. After desorption, the fiber was conditioned at 250 °C for 20 min.

Chiral separations were performed on an MDGC-MS system (Shimadzu Europa GmbH, Duisburg, Germany), which consists of two Shimadzu GC-2010 Plus gas chromatographs (*GC 1* and *GC 2*), equipped with a multi Dean's switch (MDS), a Shimadzu QP2010 Ultra mass spectrometer, and a Shimadzu AOC-20i autoinjector.

GC 1. Helium (5.0) was used as carrier gas. A polar J&W VF-WAXms column (30 m × 0.25 mm i.d. × 0.25 µm film thickness; Agilent Technologies, Waldbronn, Germany) was used for initial separation. The operational conditions were as follows: constant inlet pressure, 208.1 kPa; inlet temperature, 250 °C; splitless, 2 min; initial linear velocity, 25 cm/s; temperature program, 40 °C (3 min), raised at 5 °C/min to 220 °C (6 min). The FID (250 °C; H_2 flow, 40 mL/min; air flow, 400 mL/min; makeup gas N_2 (5.0), 30 mL/min) was connected *via* a stainless steel retention gap to the MDS.

GC 2. A BGB176 column (30% 2,3-dimethyl-6-*tert*-butyldimethylsilyl-beta-cyclodextrin dissolved in 15% phenyl-, 85% methylpolysiloxane, 30 m × 0.25 mm i.d. × 0.25 µm film thickness; BGB Analytik, Boeckten, Switzerland) was applied for the analysis of 2-methylbutanoic acid and M2MB. The oven temperature was held at 40 °C for 3 min, ramped at 1 °C/min to 150 °C, held for 3 min, increased at 20 °C/min to 200 °C, and finally held for 3 min. Further conditions were as follows: transfer line temperature between *GC 1* and *GC 2*, 200 °C; initial linear velocity, 47.2 cm/s; switching pressure, 129.1 kPa; MS mode, scan mode (*m/z*, 33–300); electron ionization energy, 70 eV; source temperature, 200 °C; quadrupole temperature, 150 °C; MS transfer line temperature, 200 °C. All data were collected using Shimadzu MDGC solution software 1.01.

2.6. Determination of odor qualities and odor thresholds of (R)-M2MB and (S)-M2MB in air

Odor thresholds in air were determined by the following procedures