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Activity-Guided Identification of (*S*)-Malic Acid 1-*O*-D-Glucopyranoside (Morelid) and γ-Aminobutyric Acid as Contributors to Umami Taste and Mouth-Drying Oral Sensation of Morel Mushrooms (*Morchella deliciosa* Fr.)

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Although morel mushrooms are widely used as tasty ingredients in savory dishes, knowledge of the key compounds evoking their attractive taste is still very fragmentary. In the present study, taste activity-guided fractionation of an aqueous morel extract by means of the recently developed taste dilution analysis (TDA) enabled the localization of several umami-like-tasting fractions as well as a fraction imparting an intense mouth-drying sensation to the oral cavity. Hydrophilic interaction liquid chromatography (HILIC), LC-MS, and amino acid analysis led to the successful identification of γ-aminobutyric acid as the chemical inducer of the mouth-drying and mouth-coating oral sensations imparted by the morel extract. Besides the well-known umami-like taste contributors L-glutamic acid, L-aspartic acid, and succinic acid, an additional HILIC fraction was isolated and evaluated as tasting umami-like. LC-MS and NMR studies revealed that this fraction consisted of a mixture of (*S*)-malic acid 1-*O*-α-D-glucopyranoside and (*S*)-malic acid 1-*O*-β-D-glucopyranoside, the structure of which could be successfully confirmed by independent synthesis. To the best of our knowledge, this morel-derived glycoside, which we named (*S*)-morelid, has previously not been reported in any food products. Sensory analysis of aqueous solutions of the compounds identified revealed threshold concentrations of 0.02 mmol/L for the mouth-drying effect of γ-aminobutyric acid and 6.0 mmol/L for the umami-like, slightly sour taste of (*S*)-morelid.

KEYWORDS: Umami; mouth-drying; mushrooms; taste dilution analysis; morelid; (*S*)-malic acid 1-*O*-β-D-glucopyranoside; γ-aminobutyric acid

INTRODUCTION

Air-dried morel mushrooms (*Morchella* spp.) are widely used as tasty ingredients in savory dishes including soups and sauces. In particular, the umami-like taste attributes and the taste-enhancing activity of the rehydrated, dried mushrooms are highly desirable and impart rich mouthfeel, complexity, thickness, and palate length to culinary products.

Although the relationship between the chemical composition and factors affecting its taste based on sensory experiments has been investigated by only a few authors (*1*−*3*), the attractive taste of mushrooms is believed to be due to a balanced interplay of soluble sugars (*4*−*20*), 5′-nucleotides (*1*, *9*−*12*), organic acids (*2*, *9*, *13*), and free amino acids (*1*, *4*, *5*, *9*, *10*, *12*, *14*, *15*) with L-glutamate as the most important umami taste contributor. Recent taste reconstitution experiments confirmed the role of L-glutamate as one of the umami-like compounds and indicated a taste-enhancing effect of the 5′-nucleotides. However, sensory comparison of the natural mushroom extract with the artificial imitation gave evidence that besides L-glutamate and purine-5′-nucleotides additional key compounds contributing to the typical umami-like taste and mouthfeel of morel extracts are not yet fully defined on a molecular level (*3*).

Besides monosodium glutamate (MSG) and some purine-5′-nucleotides such as inosine-5′-monophosphate (*16*), also hydrophilic di-, tri-, and tetrapeptides containing polar side chains, such as Glu-Asp, Glu-Glu, Thr-Glu, and Asp-Glu-Ser, have been described as eliciting a lingering umami-like taste and mouthfeel sensation (*17*−*19*). However, savory peptides are discussed controversially in the literature. Some researchers have described these di- and tripeptides as neutral or even slightly bitter and generally questioned the existence of umami peptides (*20*), whereas others have reported that tripeptides having a hydrophobic amino acid residue, such as Glu-Leu-Glu or Glu-Asp-Phe, impart mouthfeel and a glutamate-like taste to foods (*21*).

Apart from amino acids, nucleotides, and peptides, dicarboxylic acids such as succinic acid and tartaric acid exhibit some

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kind of umami-like taste (*22*, *23*). Furthermore, lactic acid was found to contribute to the glutamate-like taste of foods such as beef bouillon and stewed beef juice (*24*, *25*). Recently, *N*-lactoyl-L-glutamate, the condensation product of lactic acid and glutamic acid, was reported to evoke a bouillon-like umami taste, although weaker compared to that of MSG (*21*). Besides the N-acylation of glutamic acid, recent investigations on the Maillard reaction between glutamic acid and reducing sugars led to the discovery of the umami-like-tasting *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid (*26*).

To answer the puzzling question as to which nonvolatile, key taste compounds are responsible for the attractive taste of food products, we have recently developed the so-called taste dilution analysis (TDA) as a powerful screening procedure for taste-active nonvolatiles in foods. This approach, combining instrumental analysis and human bioresponse, led to the discovery of various previously unknown taste compounds such as thermally generated bitter compounds (*27*), cooling compounds in dark malt (*28*), bitter off-tastants in carrot products (*29*), the taste enhancer alapyridaine in beef bouillon (*30*), and astringent key taste compounds in black tea infusions (*31*).

Aimed at defining the taste of morels on a molecular level, the objectives of the present investigation were, therefore, to screen an aqueous extract prepared from dried morels for its key taste compounds by the application of taste dilution techniques, to isolate and identify the compounds inducing the most intense human taste response, and to evaluate their taste impact on the basis of their human threshold concentrations.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: silver trifluoromethane sulfonate, 1,2-dichloroethane, (*S*)-malic acid dimethyl ester, 2,6-di-*tert*-butyl-4-methylpyridine, barium hydroxide, hydrochloric acid, formic acid, sodium hydroxide, inosine-5′-monophosphate, sorbitol, mannitol (Sigma, Steinheim, Germany); α-bromotetra-*O*-acetyl-D-glucose, *β*-aminoisobutyric acid (Fluka, Taufkirchen, Germany); and Glu-Glu (Bachem, Weil am Rhein, Germany). Solvents were of HPLC grade (Merck, Darmstadt, Germany). Deuterated solvents were supplied by Euriso-Top (Gif-Sur-Yvette, France). *N*-(1-Deoxyfructos-1-yl)-L-glutamic acid was synthesized closely following the procedures reported recently (*26*).

Preparation of an Aqueous Morel Extract. Dried morel mushrooms (*Morchella deliciosa* Fr., 100 g) obtained from a local market (Munich, Germany) were ground and then soaked in drinking water (1 L) for 12 h at room temperature. The aqueous suspension was heated for 15 min under reflux prior to filtration using a cellulose filter. For sensory experiments as well as chemical analysis the aqueous morel extract was used directly after cooling.

Multistep Ultrafiltration. The freshly prepared morel extract (100 mL) was fractionated by means of an ultrafiltation cell (Amicon, Witten, Germany) using sequential filters with cutoffs of 10 kDa (YM 10), 1 kDa (YM 1), and 0.5 kDa (YC 05; Millipore, Bedford, MA) at a nitrogen pressure of 3 bar, thus yielding the four ultrafiltration fractions UF1−UF4. The individual filtrates were freeze-dried, affording fractions UF1 (5.2% in yield), UF2 (8.2% in yield), UF3 (24.4% in yield), and UF4 (62.1% in yield) as amorphous powders, which were used for instrumental analysis and sensory experiments.

Gel Permeation Chromatography (GPC). Aliquots (2 g) of the low molecular weight fraction (UF3+4; molecular mass < 1 kDa) obtained by ultrafiltration were applied onto the top of a 100 cm × 5 cm glass column (XK 50/100, Amersham Pharmacia Biotech, Freiburg, Germany) filled with an aqueous slurry of Sephadex G-15 (Amersham Pharmacia Biotech). Chromatographic separation was performed with a flow rate of 3 mL/min and was monitored at 254 nm by means of a model UV-1575 UV−vis detector (Jasco, Grossumstadt, Germany). The effluent was collected in 11 GPC fractions (I−XI), which were freeze-dried and used for TDA as well as chemical analysis.

Subfractionation of GPC Fraction III. The taste-active GPC fraction III was dissolved in water using an ultrasonic bath and was separated by means of a Strata-X cartridge (Phenomenex, Aschaffenburg, Germany), which was preconditioned with aqueous methanol (50% in water; 1 mL), followed by distilled water. After application of the sample, the cartridge was flushed with water (10 mL), and the effluent was freeze-dried and then separated by hydrophilic interaction liquid chromatography (HILIC) on a semipreparative 300 × 7.8 mm i.d. TSK-GelAmide-80 column (Tosoh BioSep, Stuttgart, Germany) equipped with a 10 × 1.4 mm guard column. Analysis was performed at a flow rate of 1.5 mL/min using a solvent mixture, A (80/20; v/v), of acetonitrile and aqueous ammonium formate (7 mM; pH 5.5), a solvent mixture, B (20/80), of acetonitrile and aqueous ammonium formate (7 mM; pH 5.5), and the following gradient: 0% B from 0 to 13 min, 0−35% B from 13 to 40 min, 35% B from 40 to 43 min. The effluent of 15 HPLC runs was separated into eight fractions (III/1−III/8), which were separately collected and freeze-dried.

TDA. GPC fractions I−XI and HILIC fractions III/1−III/8, respectively, were dissolved in exactly 2.0 mL of drinking water and were then stepwise 1+1-diluted with drinking water. The serial dilutions of each of these fractions were then presented to the sensory panel in the order of increasing concentrations, and each dilution was sensorially judged using a triangle test. The dilution at which a taste difference between the diluted fraction and two blanks (tap water) could just be detected was defined as the taste dilution (TD) factor (*27*). The TD factors evaluated by three different assessors at three different sessions were averaged.

Identification of (*S*)-Malic Acid 1-*O*-D-Glucopyranoside, (*S*)-Morelid. To remove free amino acids from HILIC fraction III/4, aliquots of that fraction were dissolved in water (20 mL), and, after adjustment of the pH to 2.0 with formic acid, were applied on a 150 × 30 mm Dowex KT WX 8 ion-exchange column (5 g; Serva, Heidelberg, Germany) conditioned with aqueous formic acid (3 mol/L; pH 2.0). After elution of the column with aqueous formic acid (10 mL; 3 mol/L), the effluent was freeze-dried, taken up in a mixture (80:20, v/v) of acetonitrile and aqueous ammonium formate buffer (7 mmol/L; pH 5.0), and then subjected to HILIC on a semipreparative 300 × 7.8 mm i.d. TSK-GelAmide-80 column (Tosoh BioSep) equipped with a 10 × 1.4 mm guard column. Analysis was performed at a flow rate of 1.5 mL/min using a solvent mixture, A (80/20; v/v), of acetonitrile and aqueous ammonium formate (7 mM; pH 5.5), a solvent mixture, B (20/80), of acetonitrile and aqueous ammonium formate (7 mM; pH 5.5), and the following gradient: 0% B from 0 to 13 min, 0−35% B from 13 to 40 min, 35% B from 40 to 43 min. The effluent of 15 HPLC runs was separated into eight fractions (III/4-1−III/4-8), which were separately collected and freeze-dried. Degustation of these fractions in water revealed a umami-like taste quality in fraction III/4-4. LC-MS and NMR experiments of the isolate led to the identification of the umami-tasting principle as a mixture of (*S*)-malic acid 1-*O*-α-D-glucopyranoside and (*S*)-malic acid 1-*O*-*β*-D-glucopyranoside.

(S)-Malic acid 1-O-α-D-glucopyranoside: HILIC/MS (ESI$^-$), *m/z* 227 (100), 295 (80), 296 (25); ^{1}H NMR (400 MHz, D_2O) *δ* 2.47 [dd, 1H, H_a-C(3′)], 2.76 [dd, 1H, H_b-C(3′)], 3.43 [m, 1H, H-C(4)], 3.47 [m, 1H, H-C(3)], 3.57 [d, 1H, Hα-C(2)], 3.74 [m, 1H, H-C(5)], 3.88 [m, 1H, H_a-C(6)], 3.90 [m, 1H, H_b-C(6)], 4.37 [d, 1H, H-C(2′)], 5.92 [d, 1H, Hα-C(1)]; ^{13}C NMR (90 MHz, D_2O) *δ* 42.5 [C(3′)), 55.0 (C(4)], 63.7 [C(6)], 70.0 [C(5)], 71.2 [C(2′)], 77.0 [Cα(2)], 77.2 [C(3)], 92.5 [Cα-(1)], 179.3 [C(1′)], 180.4 [C(4′)].

(S)-Malic acid 1-O-β-D-glucopyranoside: HILIC/MS (ESI$^-$), *m/z* 227 (100), 295 (80), 296 (25); ^{1}H NMR (400 MHz, D_2O) *δ* 2.47 [dd, 1H, H_a-C(3′)], 2.76 [dd, 1H, H_b-C(3′)], 3.30 [d, 1H, H*β*-C(2)], 3.43 [m, 1H, H-C(4)], 3.47 [m, 1H, H-C(3)], 3.74 [m, 1H, H-C(5)], 3.88 [m, 1H, H_a-C(6)], 3.90 [m, 1H, H_b-C(6)], 4.37 [d, 1H, H-C(2′)], 4.71 (d, 1H, H*β*-C^1); ^{13}C NMR (90 MHz, D_2O) *δ* 42.5 [C(3′)], 55.0 [C(4)], 63.7 [C(6)], 70.0 [C(5)], 71.2 [C(2′)], 73.8 [C*β*(2)], 77.2 [C(3)], 95.6 [C*β*(1)], 179.3 [C(1′)], 180.4 [C(4′)].

Analysis of Free Amino Acids, 5′-Nucleotides, and Soluble Carbohydrates. Following the procedure reported in the literature, the freeze-dried fractions were dissolved in the starting buffer, and free amino acids were analyzed by means of an amino acid analyzer LC 3000 (Biotronik, Maintal, Germany) with ninhydrin detection (*32*).

Organic acids were determined using enzymatic test kits (R-biopharm Boehringer, Mannheim, Germany) closely following the experimental procedures given by the manufacturers.

5′-Nucleotides were quantified by HPLC using a 250 × 4.6 mm i.d., 5 μm Phenyl-Hexyl column (Phenomenex, Aschaffenburg, Germany) as the stationary phase and an aqueous phosphate buffer (0.01 mol/L; pH 2.8) as the mobile phase (flow rate: 1 mL/min); the effluent was monitored at 254 nm by means of a UV−vis detector. Each 5′-nucleotide was identified by comparing retention times and UV−vis spectra with those of the corresponding reference substances and was quantified using inosine-5′-monophosphate as the internal standard.

Soluble carbohydrates were analyzed by means of RP-HPLC on an ET 250/4 C-18 Nucleosil 100-5 NH_2 column (Machery-Nagel, Düren, Germany) using a mixture (85:15, v/v) of acetonitrile and water as the mobile phase. Chromatography was performed at 30 °C, and separation was monitored by means of an ERC-7515A RI detector (ERC, Alteglofsheim, Germany). Each carbohydrate was identified by co-chromatography with the corresponding reference substances and was quantified using sorbitol as the internal standard.

Synthesis of (*S*)-Malic Acid 1-*O*-β-D-Glucopyranoside. Using a methodology reported in the literature for *O*-glucoside synthesis (*33*) with some modifications, an activated molecular sieve (4 Å; 4 g) was added to a suspension of silver trifluoromethane sulfonate (5.0 mmol) in anhydrous 1,2-dichloroethane (40 mL) under an argon atmosphere in the dark with stirring at room temperature. The mixture was then cooled to − 20 °C, α-bromotetra-*O*-acetyl-D-glucose (5.0 mmol) and (*S*)-malic acid dimethyl ester (2.5 mmol) were successively added, and, after 20 min of stirring at −20 °C, 2,6-di-*tert*-butyl-4-methylpyridine (5.0 mmol) was added. Finally, the cooling bath was removed, and the mixture was maintained at room temperature while stirring for 14 h. Thereafter, the solution was filtered, the solvent was removed in a vacuum, and the oily residue was fractionated by column chromatography (250 mm × 30 mm) on silica gel (silica gel 60; water content = 5%) conditioned with toluene. Chromatography with a mixture (2:1, v/v) of toluene and ethyl acetate, followed by evaporation of the solvent, yielded a pale yellow oil, which was dissolved in acetone (50 mL) and cooled to 0 °C in an ice bath. After addition of an aqueous $Ba(OH)_2$ solution (0.125 mol/L; 250 mL), the mixture was shaken for 10 min and stored for 1 h at 0 °C. After the addition of another aliquot (150 mL) of the $Ba(OH)_2$ solution (0.125 mol/L) and maintenance of the mixture for at least 3 h at 0 °C, aqueous hydrochloric acid (3 mol/L; 40 mL) was added at 0 °C, and the cooled mixture was filtered. Addition of anhydrous ethanol (500 mL) and storage at 0 °C for 12 h led to the precipitation of the title compound as the corresponding barium salt. The precipitate was isolated by membrane filtration (0.45 μm i.d.), and the residue was washed with cooled anhydrous ethanol (15 mL). Final purification and removal of the barium ions was achieved by ion exchange chromatography on a weak acidic cation-exchange resin (Mega Bond Elut CBA, 30 g) (Varian) conditioned with NaOH (0.1 mol/L; 20 mL), followed by formic acid (1.0 mol/L; 30 mL) and, finally, water (100 mL). The barium salt of the glucoside was dissolved in water and applied onto the top of the column. After the resin had been washed with water (50 mL), the eluate was freeze-dried to give (*S*)-malic acid 1-*O*-β-D-glucopyranoside (yield = 561 mg; 1.9 mmol, 76%) as a white amorphous powder: LC-MS (ESI^-), *m*/*z* 295 (100); ^1H NMR (400 MHz, D_2O) δ 2.86 [dd, 1H, H_a-C(3′)], 2.88 [dd, 1H, H_b-C(3′)], 3.30 [d, 1H, H-C(2)], 3.35 [m, 1H, H-C(4)], 3.36 [m, 1H, H-C(5)], 3.45 [m, 1H, H-C(3)], 3.67 [d, 1H, H_a-C(6)], 3.84 [dd, 1H, H_b-C(6)], 4.51 [d, 1H, H-C(1)], 4.59 [d, 1H, H-C(2′)]; ^{13}C NMR (125 MHz, D_2O) δ 38.5 [C(3′)], 60.4 [C(6)], 69.3 [C(5)], 73.1 [C(2)], 75.4 [C(3)], 75.5 [C(2′)], 75.9 [C(4)], 101.9 [C(1)], 175.0 [(C(1′)], 176.7 [C(4′)]; $[\alpha]_D^{20}$ = −3.1°.

Sensory Analyses. *Panel Training.* Using triangle tests, assessors were trained to evaluate the taste of solutions (5 mL each) of the following standard compounds: sucrose (50 mmol/L) for sweet taste; lactic acid (20 mmol/L) for sour taste; NaCl (12 mmol/L) for salty taste; caffeine (1 mmol/L) for bitter taste; and MSG (8 mmol/L, pH 5.7) for umami taste. For mouth-drying and astringency, the panel was trained by using tannin (gallustannic acid, 0.01%). Sensory analyses were performed in a sensory panel room at 19−22 °C in three different sessions.

Determination of Taste Thresholds. Taste thresholds were determined by a triangle test using tap water (pH 6.5) as solvent. The samples (3 mL) were presented in serial 1:1 dilutions in order of ascending concentrations. At the start of each session and before each trial, the subject rinsed with tap water and expectorated. The samples, blanks as well as taste solutions, were swirled in the mouth briefly and expectorated. After indicating which glass vial contained the tastant, the participant received another set of two blanks and one taste sample. To prevent excessive fatigue, tasting began at a concentration level two steps below the individual threshold concentration that had been determined in a preliminary sensory experiment. The threshold values evaluated in three different sessions by eight panelists each were averaged. The values between individuals and separate sessions differed by not more than one dilution step.

Taste Profile Analysis. The freshly prepared aqueous morel extract and the individual ultrafiltration fractions, respectively, were presented to the members of the sensory panel, who were asked to score the taste qualities umami, sour, bitter, sweet, salty, and mouth-drying on a scale from 0 (not detectable) to 3 (strongly detectable). To achieve this, the samples were swirled in the mouth briefly and expectorated.

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (BIO-TEK Kontron Instruments, Eching, Germany) consisting of two pumps (type 522), a Rheodyne injector (100 μL loop), and a UV−vis detector (type 535) was equipped with a 300 × 7.8 mm i.d. TSK-GelAmide-80 column (Tosoh BioSep) equipped with a 10 × 1.4 mm guard column.

Mass Spectrometry (LC-MS). For LC-MS analysis, the HILIC column (TSK-GelAmide-80 column, 300 × 7.8 mm i.d., 10 × 1.4 mm guard column) (Tosoh BioSep) was coupled to an LCQ mass spectrometer (Finnigan MAT GmbH, Bremen, Germany) operating in the positive (ESI^+) and negative (ESI^-) electrospray ionization modes. After injection of the sample (20−100 μL), the analysis was performed using the solvent gradient reported above. Mass spectrometry of the synthetic (*S*)-malic acid 1-*O*-β-D-glucopyranoside was performed on an API 4000 QTrap mass spectrometer (Applied Biosystems, Darmstadt, Germany) with an electrospray Turbo V−ionization interface.

Nuclear Magnetic Resonance Spectroscopy (NMR). The ^1H, ^{13}C, COSY, HMQC, and HMBC spectroscopic experiments of the glucoside isolated from morels were performed on AMX-400 and AMX-360 spectrometers (Bruker, Rheinstetten, Germany). NMR experiments of synthetic morelid were performed on an APX-400 spectrometer (Bruker) and on an Inova 500 MHz spectrometer (Varian, Darmstadt, Germany). D_2O was used as solvent and tetramethylsilane as internal standard.

Polarimetry. The optical rotation of a solution of the morelid (1% in water) was performed on a Polarimeter 341 (Perkin-Elmer, Rodgau-Jügesheim, Germany) equipped with a 100 mm cell.

RESULTS AND DISCUSSION

To obtain a representative morel extract mimicking the taste profile obtained upon following kitchen-like cooking procedures, dried morel mushrooms were soaked in water overnight and then heated for 15 min under reflux. The freshly prepared aqueous extract obtained after cooling and filtration imparted the typical complex morel taste and was used for taste profile analysis. To achieve this, the trained sensory panel was asked to rate the intensity of the taste qualities given in **Figure 1** on a scale from 0 (not detectable) to 3 (intensely detectable). By far the highest scores were found for the intensity of the umami (2.1) as well as sour taste sensation (1.6), followed by a mouth-drying sensation, evaluated with a somewhat lower intensity of 1.3. In comparison, the taste qualities bitter, sweet, and salty were rated with very low intensities.

To gain first insight into the chemical compounds imparting the intense umami taste as well as the mouth-drying sensation perceived in the oral cavity, the influence of the molecular weight of the mushroom constituents on their contribution to the overall taste of the morel extract was investigated. To achieve this, the freshly prepared morel extract was fractionated

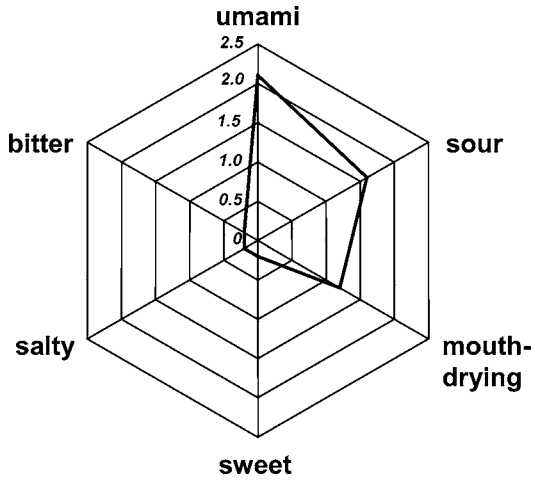

Figure 1. Taste profile analysis of a freshly prepared, aqueous morel extract.

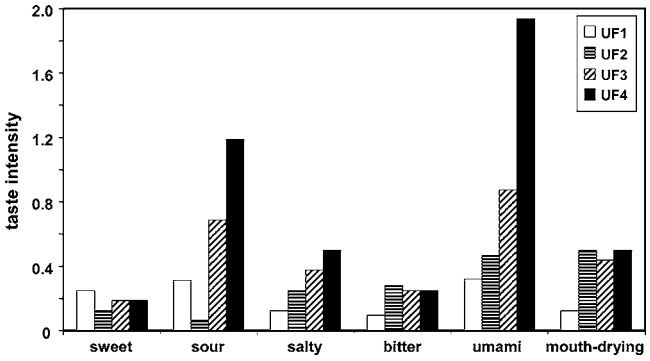

Figure 2. Influence of the molecular weight of morel fractions on the intensity of individual taste qualities.

by means of a multistep ultrafiltration. Using filters with cutoffs of 10, 1, and 0.5 kDa in sequence, the four fractions UF1−UF4 were obtained. The deep brown fraction UF1 contained compounds with molecular masses above 10 kDa, fraction UF2 containing compounds with molecular masses between 1 and 10 kDa showed a brown coloration, the light brown fraction UF3 contained the components with molecular masses between 1 and 0.5 kDa, and the yellowish fraction UF4 contained the compounds with molecular masses below 0.5 kDa. After freeze-drying, each individual fraction was dissolved in water in its "natural" concentration and the sensory impressions umami-like, sourness, bitterness, saltiness, and sweetness as well as the mouth-drying sensation were then rated by their intensities through application of the taste profile analysis. Although intensely brown in color, fraction UF1 was nearly tasteless and showed only a very faint taste sensation judged only with intensity scores below 0.3 (**Figure 2**). In comparison, the low molecular weight fraction UF4 was described by the sensory panel to impart the typical complex taste profile of the morel extract and was evaluated with high intensities for the umami (1.9) and sour (1.2) taste modalities. Also, fraction UF3 induced a umami sensation in the oral cavity, but its intensity was significantly lower than that detected in fraction UF4. In addition, the mouth-drying sensation with an intensity of 0.5 was detected in fractions UF2−UF4 (**Figure 2**). As these data showed the compounds with molecular mass below 1 kDa as the main contributors to the typical morel taste, the following tastant mapping was focused on the recombined fraction UF3+4.

Mapping of Key Morel Taste Compounds. To locate the key taste compounds in the morel extract, the low molecular

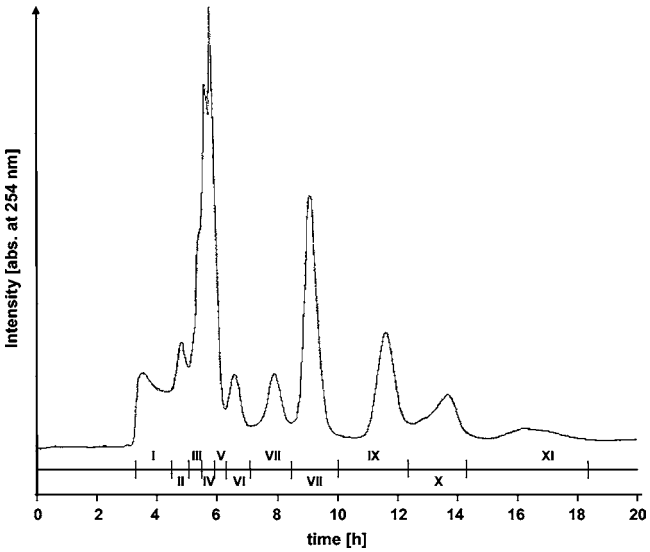

Figure 3. GPC chromatogram of the low molecular weight fraction UF 3+4 (molecular mass < 1 kDa) isolated from morel extract by means of ultrafiltration.

Table 1. Taste Dilution Analysis of GPC Fractions Isolated from the Low Molecular Weight Fraction (UF3+4; Molecular Mass < 1 kDa) of Morel Extract

fraction[a]	taste quality[b]	TD factor[c]	fraction	taste quality	TD factor
I	astringent	1	VI	astringent	4
II	sour	4		mouth-coating	2
	umami	4	VII	astringent	1
III	mouth-drying	64	VIII	astringent	1
	umami	32	IX	astringent	1
	sour	16	X	astringent	1
IV	sour	8	XI	tasteless	<1
	umami	4			
	astringent	4			
	sweet	1			
V	sweet	2			
	bitter	2			
	umami	2			
	astringent	2			

[a] Number of GPC fractions refers to **Figure 3**. [b] Taste quality was determined by a trained sensory panel. [c] Data are given as the mean of the TD factors evaluated by five different assessors in three different sessions.

weight fraction UF3+4 was further analyzed by GPC using Sephadex G-15 as the stationary phase and water as the mobile phase (**Figure 3**). The chromatography was monitored at 254 nm, and the effluent was separated into 11 fractions (I−XI), which were individually freeze-dried and then dissolved in the same amount of water. The aqueous solution of each individual fraction was then presented to the sensory panel, who was asked to judge the taste qualities and intensities by application of the TDA. To achieve this, each solution was stepwise 1+1 diluted with water, and the dilutions were then presented in order of increasing concentrations to trained sensory panellists, who were asked to evaluate the taste quality and to determine the dilution at which a taste difference between the diluted fraction and two blanks (tap water) could just be detected in a triangle test. As this so-called TD factor, obtained for each fraction, is related to its taste activity in water, the 11 GPC fractions were rated according to their relative taste impact (**Table 1**). GPC faction III was evaluated with by far the highest taste impact and showed a complex taste centering around mouth-drying, umami, and sour notes, which were evaluated with the highest TD

Table 2. Concentrations of Free Amino Acids, Nucleotides, and Soluble Carbohydrates in Individual GPC Fractions Isolated from the Low Molecular Weight Fraction (UF3+4) of Morel Extract

compound	taste quality	amount (μmol) in GPC fraction[a] I	II	III	IV/V	VI	VII	VIII	IX	X
amino acids										
L-glutamic acid	umami		24	190	12					
L-aspartic acid	umami			62						
L-proline	sweet	1	2	10	8					
L-threonine	sweet			28	40					
L-serine	sweet			18	59					
L-alanine	sweet			85	2					
glycine	sweet				48					
L-lysine	bitter	2	39	3						
L-arginine	bitter	5	1	42						
L-valine	bitter	2	2	22	1					
L-isoleucine	bitter			2	47					
L-histidine	bitter			7	5					
L-leucine	bitter				62					
L-tyrosine	bitter				1	2	4	22		
L-phenylalanine	bitter						23			
L-cysteine	sulfury	7								
L-methionine	sulfury	2	4	3	13					
nucleotides										
AMP	umami					6				
UMP	umami					10				
hypoxanthine	bitter						10			
carbohydrates										
mannitol	sweet			3	45					
glucose	sweet				41					

[a] The concentrations of the taste compounds have been determined in the GPC fractions obtained from the low molecular weight fraction (UF3+4) isolated from 5.7 g of dried morels.

factors of 64, 32, and 16. In addition, a sweet taste was detectable in fractions IV and V with a TD factor of 2, and a bitter taste was rated with a TD factor of 2 in fraction V. Furthermore, fractions IV−XI imparted a weak astringent sensation to the oral cavity. To clarify the questions as to which of the well-known taste-active amino acids, soluble carbohydrates, and nucleotides are contributing to the taste of the individual GPC fractions, the concentrations of these potential taste compounds were determined in the taste-active fractions I−X.

First, free amino acids were quantified in the individual GPC fractions using an amino acid analyzer with ninhydrin detection. As given in **Table 2**, most of the amino acids were detectable in fractions II−V. The major amount of the umami-like-tasting amino acids glutamic acid and aspartic acid was located in fraction III, which was evaluated with a high TD factor for umami taste (cf. **Table 1**). The major amount of the sweet-tasting amino acids eluted in fractions III−V, whereas bitter-tasting amino acids were found to be widely separated in fractions I−VIII. RP-HPLC analysis of nucleotides revealed that just the umami-like-tasting and taste-enhancing adenosine-5′-monophosphate (AMP) and uridine-5′-monophosphate (UMP) as well as the bitter-tasting hypoxanthine were present in significant amounts in the morel extract; both 5′-ribonucleotides coeluted in GPC fraction VI, whereas the bitter hypoxanthine was detected in fraction VII (**Table 2**). HPLC analysis of the soluble carbohydrates in the morel extract led to the identification of glucose and mannitol, which were detected mainly in the GPC fractions IV and V, thus corresponding well with the sweet taste detected for these fractions (cf. **Table 1**).

With the aim of investigating the chemical nature of the mouth-drying substance in morel extract and studying the potential existence of hitherto unknown umami-like taste compounds in GPC fraction III, this GPC fraction needed to be

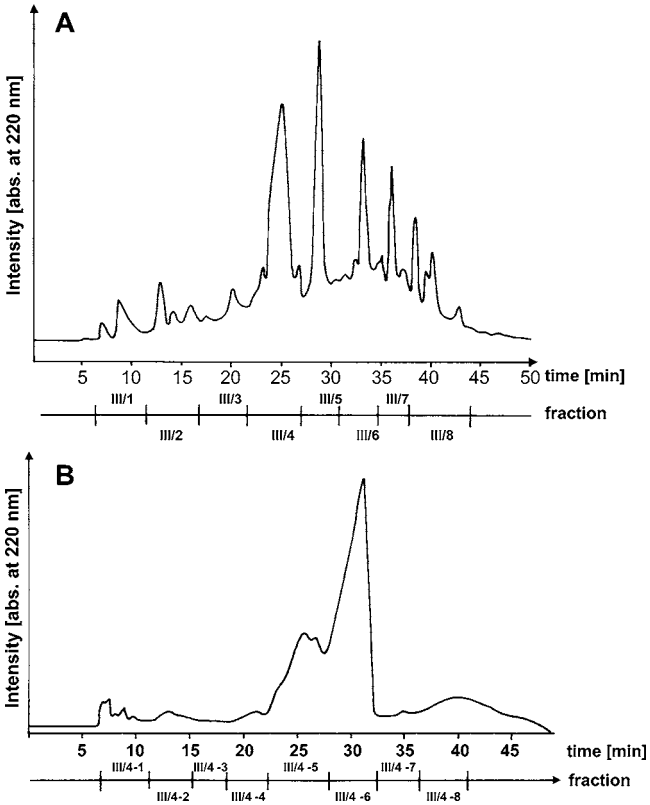

Figure 4. HILIC chromatogram of (**A**) GPC fraction III isolated from morel extract by means of ultrafiltration and (**B**) rechromatography of HILIC subfraction III/4.

Table 3. Taste Dilution Analysis of HILIC Fractions Isolated from GPC Fraction III

fraction[a]	taste quality[b]	TD factor[c]	fraction	taste quality	TD factor
III/1	tasteless	<1	III/5	mouth-drying	8
III/2	sour	8		umami	4
	umami	4		sweet	4
III/3	bitter	2	III/6	sweet	4
	sour	2		umami	2
III/4	umami	16	III/7	umami	32
	sweet	1	III/8	umami	4

[a] Number of HILIC fractions refers to **Figure 4A**. [b] Taste quality was determined by a trained sensory panel. [c] Data are given as the mean of the TD factors evaluated by five different assessors in three different sessions.

resolved into individual compounds in order to rate them according to their relative taste impacts. HPLC analysis on an RP-18 stationary phase did not, however, allow a successful separation of the compounds in GPC fraction III (data not shown). Therefore, fraction III was first preseparated into polar and hydrophobic fractions by means of a Strata-X column, and both fractions were evaluated sensorially by degustation in aqueous solution. As the typical mouth-drying as well as the umami sensation was perceived just in the polar fraction, HILIC was used to further resolve the polar compounds into eight subfractions (**Figure 4A**). After freeze-drying, subfractions III/1−III/8 were taken up in exactly the same amount of water and were then used for TDA (**Table 3**). The highest TD factor was found for HILIC fraction III/7, in which the umami note was perceived even when the original fraction was diluted by a factor of 32 (**Table 3**). More interestingly, also the fractions III/2, III/4, and III/8 imparted umami taste judged with high TD factors of 16 or 4, respectively, thus indicating the existence

Table 4. Concentrations of Free Amino Acids Identified in the Individual HILIC Fractions Isolated from the GPC Fraction III of the Morel Extract

amino acid	taste quality	amount (μmol) in HILIC fraction[a]							
		III/1	III/2	III/3	III/4	III/5	III/6	III/6	III/8
L-isoleucine	bitter		1	6					
L-leucine	bitter		4	2					
L-valine	bitter			16	1				
L-histidine	bitter								2
L-arginine	bitter								2
L-threonine	sweet				1	2	14		
L-alanine	sweet				16	20	18		
L-serine	sweet						10	7	
glycine	sweet						8		
L-aspartic acid	umami							9	
L-glutamic acid	umami							9	

[a] Amino acid concentrations were determined in the individual HILIC fractions isolated from GPC fraction III prepared from the low molecular weight fraction (UF3+4) corresponding to 5.7 g of dried morels.

of different chemical species for the umami taste. In addition, the compound inducing the intense mouth-drying sensation could be successfully located in HILIC fraction III/5 with a TD factor of 8.

Identification of the Mouth-Drying Principle. To elucidate the structure of the mouth-drying compound, HILIC fraction III/5 was further analyzed by an amino acid analyzer as well as by LC-MS. The amino acid analysis revealed the presence of the sweet-tasting L-alanine and L-threonine in fraction III/5, thus being well in line with the sweet taste perceived for this fraction (**Table 3**). In addition, another ninhydrin-positive substance was detected, which did not belong to the proteinogeneous amino acids. Analysis of that fraction by means of HILIC-MS in the ESI^+ mode revealed *m*/*z* 104 as the pseudomolecular ion $[M + 1]^+$ and indicated a molecular mass of 103 Da for the taste compound. By application of HILIC-MS as well as amino acid analysis using nonproteinogeneous amino acids as standards, this mouth-drying substance could be unequivocally identified as γ-aminobutyric acid. To the best of our knowledge, the mouth-drying sensation of γ-aminobutyric acid has yet not been reported in the literature.

Identification of Umami-like-Tasting Compounds. To investigate the chemical species responsible for the umami taste of fractions III/2, III/4, and III/7 and to identify the fraction containing the umami-like-tasting glutamic acid and aspartic acid, first, the HILIC fractions were analyzed by means of the amino acid analyzer. The amounts of the amino acids in the individual HILIC fractions are summarized in **Table 4**. L-Glutamic acid and L-aspartic acid both eluted in fraction III/7, which was judged with the high TD factor of 32 for its umami taste (cf. **Table 3**).

Although fraction III/2 showed a umami taste evaluated with a TD factor of 4 (**Table 3**), just L-leucine and L-isoleucine, but no umami-tasting amino acid, were detectable in that fraction. Enzymatic analysis of organic acids revealed that succinic acid was present in major amounts in fraction III/2. Sensory analysis of an aqueous solution of succinic acid revealed a sour taste followed by a distinct umami-like taste impression. Taste threshold concentrations were determined for the succinic acid by means of a triangle test and found to be 0.7 and 0.9 mmol/L for the umami-like and sour taste qualities, respectively.

Fraction III/4 also showed an intense umami taste evaluated with a TD factor of 16 (**Table 3**), but just the amino acids L-alanine, L-valine, and L-threonine (**Table 4**) and no additional organic acids were detectable in that fraction. To study whether these amino acids already give a umami note, an aqueous solution containing these amino acids in their "natural" concentrations was sensorially evaluated. The combination of these amino acids revealed only a slight sweet note, rather than a umami taste. To elucidate whether the umami taste of fraction III/4 is imparted by other umami compounds already reported in the literature, this fraction was spiked with the synthetic reference of *N*-(1-deoxyfructos-1-yl)-L-glutamic acid (*26*) as well as the dipeptide Glu-Glu and was analyzed by HILIC coupled to a mass spectrometer using exactly the same conditions as used for the separation of GPC fraction III. The HILIC/MS analysis revealed that these umami-like-tasting reference compounds eluted from the column after glutamic acid (t_R = 36 min) with retention times of 37 and 38 min, respectively. These findings clearly demonstrated that none of these compounds is imparting the umami taste sensation in fraction III/4 and indicated the existence of an unknown, umami-like compound in fraction III/4.

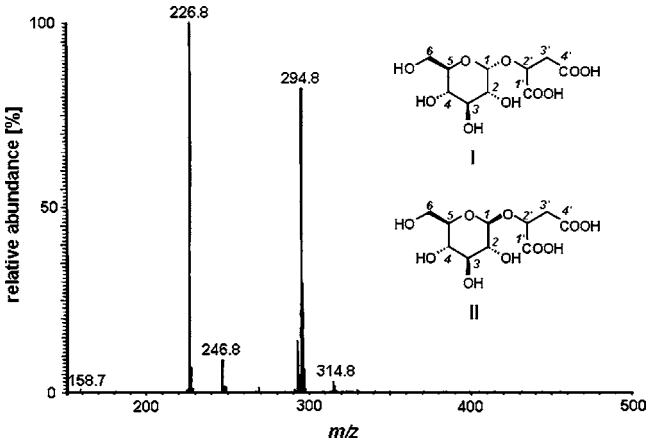

Figure 5. LC-MS spectrum of (*S*)-malic acid 1-*O*-α-D-glucopyranoside (**I**) and (*S*)-malic acid 1-*O*-β-D-glucopyranoside (**II**) isolated from fraction III/4-4.

Because fraction III/4 still consisted of a multiplicity of compounds such as free amino acids, this fraction was further separated by cation-exchange chromatography. Semipreparative HILIC analysis of the effluent obtained allowed the separation of the acidic compounds into eight subfractions, III/4-1−III/4-8 (**Figure 4B**), which were rated for their taste impact using the TDA. The highest umami-like taste impact was found for fraction III/4-4, judged with a TD factor of 8, followed by fractions III/4-5 and III/4-6 with somewhat lower TD factors. The other fractions did not impart any taste. To enable the structure determination of the umami compound, fraction III/4-4 was isolated from 30 g of dried morel mushrooms and analyzed by LC-MS and NMR experiments.

Analysis of the fraction H-IV-4 by LC-MS in the negative electrospray ionization mode revealed an $[M - H]^-$ ion with *m*/*z* 295, thus indicating a molecular mass of 296 Da (**Figure 5**). Being well in line with the mass spectrometric data, 1D- and 2D-NMR experiments led to the identification of the taste-active isolate as a 77:23 mixture of (*S*)-malic acid 1-*O*-α-D-glucopyranoside and (*S*)-malic acid 1-*O*-β-D-glucopyranoside. In detail, the ^1H NMR spectrum showed 11 resonance signals, 9 signals integrated for one proton and the residual 2 signals integrated for 0.23 and 0.77 proton equiv. The two geminal protons H_a-C(3′) and H_b-C(3′) could be assigned due to the large coupling constant of 14.6 Hz. This was further confirmed by means of homonuclear H,H correlation spectroscopy (COSY), which showed an additional coupling of these two protons with the proton H-C(2′), resonating at 4.37 ppm. ^{13}C NMR spec-

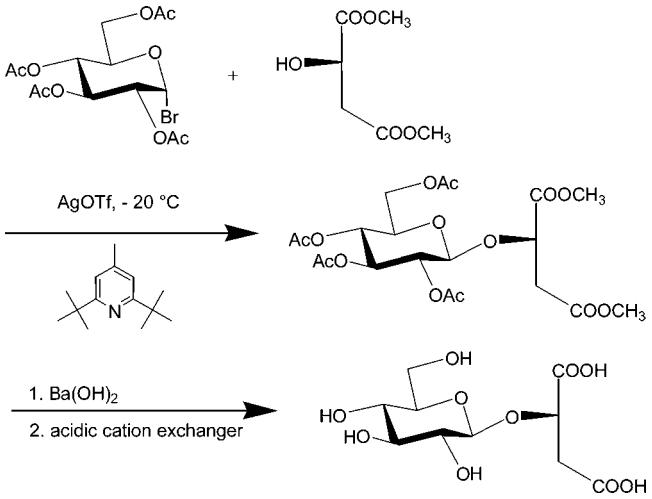

Figure 6. Reaction scheme used for the synthesis of (*S*)-malic acid 1-*O*-*β*-D-glucopyranoside, (*S*)-morelid.

troscopy showed 10 resonance signals, among which the two carbon atoms resonating at 179.3 and 180.4 ppm corresponded to the two carboxylate groups of the malic acid moiety. Heteronuclear multiple quantum correlation spectroscopy (HMQC) optimized for $^{1}J_{C,H}$ coupling constants and heteronuclear multiple bond correlation spectroscopy (HMBC) optimized for $^{2}J_{C,H}$ and $^{3}J_{C,H}$ coupling constants revealed a $^{2}J_{C,H}$ correlation between the methylene protons H_a-C(3′) and H_b-C(3′) and the carbon atom C(4′) resonating at 180.4 ppm as well as between the proton H-C(2′) and the carbon atom with a chemical shift of 179.3 ppm. The anomeric proton of the α-epimer and the *β*-epimer of the glucoside showed heteronuclear correlation with the carbon resonance signals at 92.5 and 95.6 ppm, respectively. On the basis of these findings, all of the other proton shifts of the sugar skeleton in (*S*)-malic acid 1-*O*-α-D-glucopyranoside and (*S*)-malic acid 1-*O*-*β*-D-glucopyranoside could be successfully assigned by means of the COSY experiment.

To confirm the proposed structure of the taste compounds and to obtain enough material for enabling sensory experiments, (*S*)-malic acid 1-*O*-*β*-D-glucopyranoside was synthesized by reacting (*S*)-malic acid dimethyl ester with α-bromotetra-*O*-acetyl-D-glucose using silver trifluoromethane sulfonate as the catalyst (**Figure 6**). After deprotection with barium hydroxide, the barium salt of the title compounds was precipitated upon addition of ethanol, after removal of the barium ions by means of ion-exchange chromatography; the synthetic product was analyzed by LC-MS and NMR spectroscopy as well as the sensory panel. The synthetic reference showed spectroscopic (LC-MS, NMR), chromatographic (HILIC), and sensory data identical withthose of the (*S*)-malic acid 1-*O*-*β*-D-glucopyranoside isolated from fraction III/4-4 obtained from the morel extract. To the best of our knowledge, this glycoside, which we named (*S*)-morelid, has previously not been reported in the literature as a taste-active food ingredient.

Sensory Activity of *γ*-Aminobutyric acid and (*S*)-Morelid. Sensory experiments demonstrated that aqueous solutions of *γ*-aminobutyric acid exhibited a slightly sour taste besides the typical mouth-drying and mouth-coating sensation. To evaluate the sensory impact of this amino acid, the taste threshold for the mouth-drying sensation was determined in water by means of a triangle test. The recognition threshold of that mouth-drying sensation was found to be 0.02 mmol/L. To gain first insights into the influence of the pH value on the sensory activity of *γ*-aminobutyric acid, sensory analyses were performed with aqueous solutions of the amino acid (0.1 mmol/L) adjusted to pH 3.5, 5.0, 6.0, and 7.5 (**Figure 7**). Being well in agreement with the taste thresholds of that compound in drinking water (pH 7.0), weak acidic to alkaline conditions (pH 6.0−7.5) favored the mouth-drying oral sensation, whereas the intensity of the sour taste impression was significantly weaker. Lowering the pH value to 5.0 resulted in similar intensities of the mouth-drying and the sour oral sensation, whereas at pH 3.5 the sour taste impression was predominant (**Figure 7**).

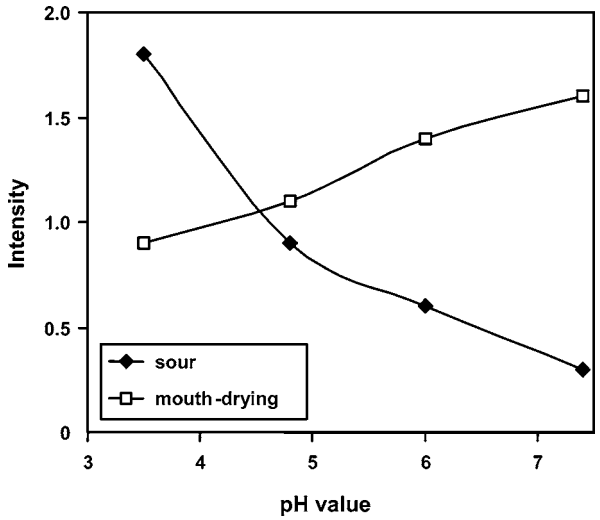

Figure 7. Influence of pH value on perceived intensity of sourness as well as mouth-drying sensation imparted by aqueous solutions of *γ*-aminobutyric acid (0.1 mmol/L).

Table 5. Taste Qualities and Taste Threshold Concentrations of *γ*-Aminobutyric Acid Homologues

amino acid	taste qualitiy	threshold concn[a] (mmol/L)
α-aminoacetic acid (glycine)	sweet	30
β-aminopropanoic acid (*β*-alanine)	sweet	1200
γ-aminobutyric acid	mouth-drying	0.02
β-aminoisobutyric acid	mouth-drying	0.12
δ-aminovaleric acid	tasteless	>1000
ϵ-aminohexanoic acid	tasteless	>1000

[a] Taste recognition threshold concentrations have been determined in water by means of triangle tests. Results are given as the mean of the data evaluated by eight different assessors in three different sessions.

To further investigate the relationship between the chemical structure and mouth-drying activity of *γ*-aminobutyric acid, aqueous solutions of the homologous amino acids α-aminoacetic acid (glycine), *β*-aminopropanoic acid (*β*-alanine), *β*-aminoisobutyric acid, *δ*-aminovaleric acid, and *ϵ*-aminohexanoic acid were evaluated by the trained sensory panel, and taste threshold concentrations were determined by means of triangle tests. As summarized in **Table 5**, exclusively the *γ*-aminobutyric acid exhibited the mouth-drying oral sensation at the extraordinarily low recognition threshold concentration of 0.02 mmol/L. In comparison, *β*-aminoisobutyric also imparted a mouth-drying sensation to the oral cavity, but its recognition threshold of 0.12 mmol/L was ∼6-fold above the threshold found for the *γ*-aminobutyric acid. In contrast, glycine and *β*-alanine imparted a sweet taste at threshold concentrations of 30 and 1200 mmol/L, respectively. It is interesting to note that the insertion of one or two further methylene groups between the carboxy function and the amino group of *γ*-aminobutyric acid revealed a loss of any taste activity; for example, *δ*-aminovaleric acid and *ϵ*-aminohexanoic acid did not impart any sensation to the oral cavity (**Table 5**).

To further investigate the sensory activity of (*S*)-morelid, the human sensory recognition threshold of the purified (*S*)-malic acid 1-*O*-*β*-D-glucopyranoside was determined in water. Using a triangle test, the threshold concentration for the umami-like, slightly sour taste of the glucoside was found to be 6 mmol/L (water). A comparative taste profile analysis of a 20 mM aqueous solution of (*S*)-morelid and a 4 mM aqueous solution of MSG showed similar intensities for the umami-like taste. The oral evaluation of the glycoside did, however, not show any salty note as compared to MSG. Aimed at demonstrating the contribution of (*S*)-morelid to the typical taste of morel extracts, quantitative studies, followed by taste reconstitution and taste omission experiments, are currently in progress and will be published elsewhere.

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