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Activity-Guided Discovery of (*S*)-Malic Acid 1′-*O*-*β*-Gentiobioside as an Angiotensin I-Converting Enzyme Inhibitor in Lettuce (*Lactuca sativa*)

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ABSTRACT: Angiotensin-converting enzyme (ACE), playing a crucial role in the renin angiotensin aldosterone system, is well-known to catalyze the conversion of the decapeptide angiotensin I into the physiologically active octapeptide angiotensin II, triggering blood pressure increasing mechanisms. To meet the demand for natural phytochemicals as antihypertensive agents in functional food development, extracts prepared from a series of vegetables were screened for their ACE-inhibitory activity by means of a LC-MS/MS-based in vitro assay. By far the highest ACE inhibition was found for a lettuce extract, in which the most active compound was located by means of activity-guided fractionation. LC-MS, NMR spectroscopy, and hydrolysis experiments followed by ion chromatography led to the unequivocal identification of the ACE inhibitor as the previously not reported (*S*)-malic acid 1′-*O*-*β*-gentiobioside. This glycoside represents a novel class of ACE-inhibiting phytochemicals with a low IC_{50} value of 27.8 *μ*M. First incubation experiments in saliva and aqueous hydrochloric acid demonstrated the stability of (*S*)-malic acid 1′-*O*-*β*-gentiobioside against salivary glycosidases and stomach acid.

KEYWORDS: *angiotensin-converting enzyme, ACE, blood pressure, hypertension, (S)-malic acid 1′-O-β-gentiobioside*

INTRODUCTION

Today, about one-fourth of the world's adult population is afflicted by hypertension, a progressive dysfunction bearing on several chronic diseases such as diabetes and cardiovascular and renal diseases.[1] Although dietary and lifestyle changes are recommended to improve blood pressure control, additional drug treatment is required to decrease the risk of associated health complications.

The pathogenesis of hypertension seems to be affected by various factors such as the increased activity of the renin angiotensin aldosterone system (RAAS), the kalikererenin kinin system, and sympathetic nervous system, as well as the complex interplay of genetic and environmental issues, respectively.[2,3] Within the RAAS enzyme cascade, angiotensinogen produced in the liver is cleaved by renin to release the inactive decapeptide angiotensin I. The angiotensin-converting enzyme (ACE), a zinc-containing dipeptidyl carboxypeptidase, cleaves histidyl-leucine from angiotensin I to give the physiologically active octapeptide angiotensin II, exhibiting vasoconstricting activity and inducing blood pressure increase. Therefore, inhibition of ACE is considered to be a promising therapeutic target to control overexpression of RAAS. Today, about 35% of hypertensive patients are treated with ACE inhibitors alone and 55% with a combinatorial medication containing an ACE inhibitor.[4]

Compared to the plethora of synthetic antihypertensive drugs, surprisingly little information is available on ACE-inhibitory natural products in our daily diet. Inspired by the introduction of an in vitro assay for the detection of ACE inhibitors,[5,23] flavonoids such as proanthocyanidines were reported in plant extracts to exhibit some ACE-inhibitory activity.[6−8,22,24] Moreover, oligopeptides isolated from digests of plant and animal proteins were found to be potent ACE inhibitors.[9−19] Among these, the dipeptide L-isoleucyl-L-tryptophan was reported as the most potent food-derived ACE inhibitor, exhibiting an IC_{50} value of 0.7 *μ*mol/L.[19] However, these peptides were found in rather low concentrations and were reported to be readily cleaved by proteases in the gastrointestinal (GI) tract and, therefore, show only rather low in vivo activities.[20] In consequence, there is still a huge demand for alternative, naturally occurring lead structures to be used as antihypertensive agents in functional food development.

As the world of plant-derived foods is the main dietary source for chemically diverse natural products, the objective of the present study was to screen vegetable extracts for their ACE-inhibitory potential by means of an in vitro assay, to locate the active ingredients in the most active extract by means of activity-guided fractionation, and to determine the chemical structure of the most active ACE inhibitor by means of LC-MS/MS and 1D/2D-NMR spectroscopy.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: formic acid and hydrochloric acid (Grüssing, Filsum, Germany); HPLC grade solvents and Tris-HCl (Merck, Darmstadt, Germany); deuterium oxide (Euriso-Top, Gif-Sur-Yvette, France); angiotensin-converting enzyme (ACE) from rabbit lung, captopril, hippuryl histidyl leucine, and hippuric acid (Sigma-Aldrich, Steinheim, Germany). Fresh samples of leek (*Allium ampeloprasum* subsp. *ampeloprasum*), lettuce (*Lactuca sativa* var. *capitata*), parsley (*Petroselinum crispum* subsp. *crispum*), fennel (*Foeniculum vulgare* var. *azoricum*), Swiss chard (*Beta*

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vulgaris subsp. *vulgaris* var. *vulgaris*), celery stalks (*Apium graveoleus* var. *dulce*), garlic (*Allium sativum*), pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*), onion (*Allium cepa*), and white cabbage (*Brassica oleracea* var. *capitata* f. *alba*) were purchased from a local vegetable store.

Preparation of Vegetable Extracts. Samples (100 g) of each vegetable were cut into small pieces, immediately frozen with liquid nitrogen, lyophilized, and then ground in a laboratory mill. Aliquots (3.5 g) of the powdered material were extracted twice with a methanol/water mixture (70:30, v/v; 200 mL) adjusted to pH 5.0 with aqueous formic acid (1 mmol/L). After centrifugation (10500 rpm) for 20 min at 10 °C, the combined extracts of each vegetable were concentrated under vacuum, freeze-dried twice, and kept at −18 °C until used for further analysis.

In Vitro Assay for ACE Inhibition. Following a literature protocol with some modifications,[14] aliquots (50 μL) of the ACE solution (2.5 mU ACE in 50 mM Tris-HCl buffer solution, pH 8.3) were preincubated at 37 °C with solutions (50 μL; 1.0 mg/mL) of the freeze-dried vegetable extracts in buffer (50 mM Tris-HCl buffer solution, pH 8.3), solutions of chromatographic fractions in their "natural" concentration ratios, or a reference solution of captopril (6.95 μg/L in 50 mM Tris-HCl buffer solution, pH 8.3), respectively. After incubation for 30 min, an aliquot (50 μL) of a solution of hippuryl L-histidyl-L-leucine (3 mmol/L in 50 mM Tris-HCl buffer solution, pH 8.3) was added and, after homogenization, was incubated at 37 °C. After 60 min, the enzymatic reaction was terminated by the addition of aqueous hydrochloric acid (50 μL, 0.5 mol/L), and aliquots (5 μL) were analyzed by means of HPLC-MS/MS on a 150 × 2 mm, 5 μm, Synergi Fusion RP-80 column (Phenomenex, Aschaffenburg, Germany) equipped with a guard column of the same type. Using the multiple-reaction monitoring (MRM) mode, hippuric acid was detected by recording the characteristic mass transition m/z 177 → 133 after collision-induced dissociation. Method validation revealed linearity between 0.5 and 4.5 mg/L, and the average coefficient of variation was below 10%. The half-maximum inhibitory concentration (IC_{50}) was determined from the data obtained for ACE inhibition of a fraction or a test compound at 10 concentration levels (up to 50 mg/100 mL) as exemplified in Figure 1. The IC_{50} value of the synthetic drug captopril was determined with 0.017 μmol/L, matching well the data found in the literature.[4,9] Data are given as the means of triplicates.

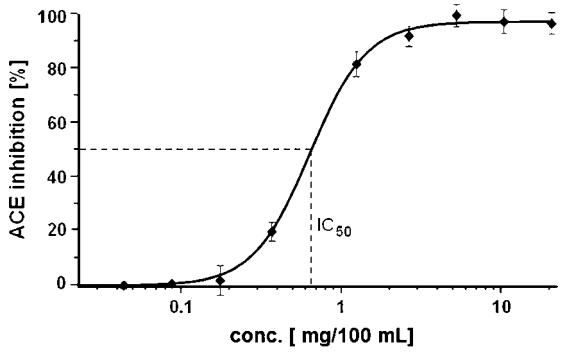

Figure 1. Dose−response curve of ACE inhibition of lettuce fraction I. IC_{50} value indicated the concentration of half-maximum inhibition.

Fractionation of Lettuce Extract by Means of RP18 Chromatography. An aliquot (1.0 g) of the lettuce extract was dissolved in water (10 mL) and placed onto the top of a water-cooled 21 × 3 cm i.d. glass column filled with a slurry of Microsorb-MV 100-5 C18 material (Varian, Darmstadt, Germany) in water and connected to a type P1 peristaltic pump (Amersham Pharmacia Biotech, Freiburg, Germany). Using a flow rate of 2 mL/min, chromatography was performed using water (200 mL; fraction I), followed by water/acetonitrile (95:5, v/v; 200 mL; fraction II), water/acetonitrile (90:10, v/v; 200 mL; fraction III), and water/acetonitrile (50:50, v/v; 200 mL; fraction IV). Separation of the solvent under vacuum, followed by freeze-drying, revealed fractions I−IV as amorphous powders in yields of 88, 6, 4, and 2%, respectively. The fractions were kept at −18 °C until further analysis.

Subfractionation of Lettuce Fraction I by Means of Hydrophilic Interaction Liquid Chromatography (HILIC). An aliquot of lettuce fraction I was taken up in a mixture (75:25, v/v) of acetonitrile and aqueous formic acid (0.1% in water) and separated by means of semipreparative HILIC on a 300 × 21.5 mm i.d., 10 μm, TSK-gel Amide-80 column (Tosoh Bioscience, Stuttgart, Germany) using a flow rate of 6 mL/min. Chromatography was performed with a mixture (75:25, v/v) of acetonitrile as solvent A and aqueous formic acid (0.1% in water) as solvent B for 40 min, increasing the content of solvent B to 75% within 20 min, then to 90% within an additional 30 min, and, finally, to 100% within 5 min. The effluent was separated into six subfractions, namely, fractions I/1−I/6 (Figure 2), which were collected from several HPLC runs, freed from solvent under vacuum, and freeze-dried three times.

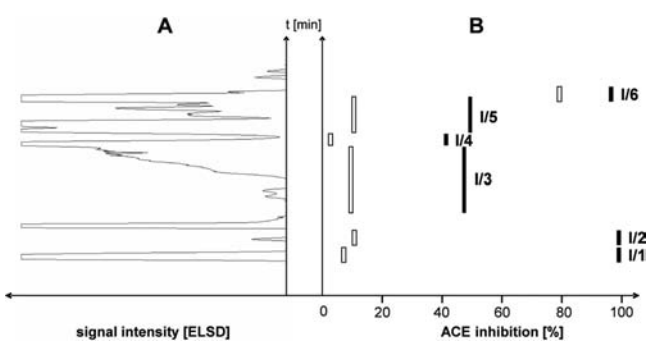

Figure 2. (A) HILIC-ELSD chromatogram of lettuce fraction I/6 and (B) ACE-inhibitory activity of each fraction before (black bars) and after (white bars) dilution with water (1:5, v/v).

Isolation of (*S*)-Malic Acid 1′-*O*-β-Gentiobioside (1) from Fraction I/6. Rechromatography of fraction I/6 was performed on a 300 × 21.5 mm i.d., 10 μm, TSK-gel Amide-80 HILIC column (Tosoh Bioscience). Using a flow rate of 6 mL/min, separation was done with a mixture (95:5, v/v) of acetonitrile and aqueous formic acid (0.1% in water) for 10 min, followed by an increase of the content of aqueous formic acid to 100% within 80 min. The effluent was separated into five subfractions, namely, fractions I/6a−I/6e, which were collected from several HPLC runs, freed from solvent under vacuum, and freeze-dried three times. ^{1}H NMR (Figure 3), ^{13}C NMR, and LC-MS/MS experiments enabled the structure determination of the ACE inhibitor in lettuce fraction I/6e as the previously not reported (*S*)-malic acid 1′-*O*-β-gentiobioside (**1**).

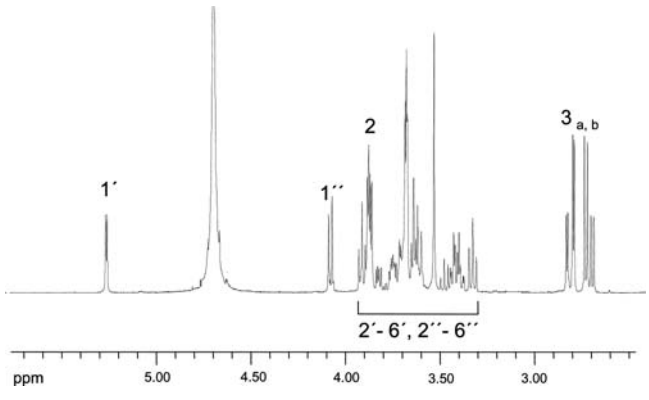

Figure 3. Excerpt of the ^{1}H NMR spectrum (400 MHz, D_2O) of (*S*)-malic acid 1′-*O*-β-D-gentiobioside (**1**).

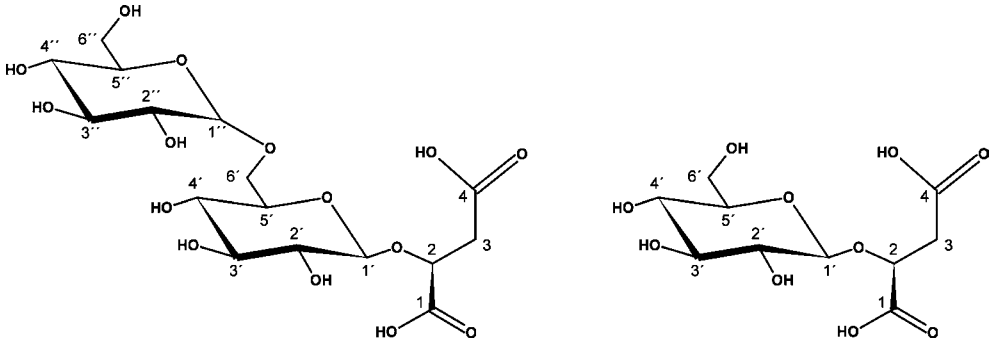

Figure 4. Chemical structures of (*S*)-malic acid 1′-*O*-*β*-D-gentiobioside (**1**) and (*S*)-malic acid 1′-*O*-*β*-D-glucoside (**2**).

*(S)-Malic acid-1′-O-β-gentiobioside (**1**, Figure 4):* LC-MS (ESI^-) *m*/*z* (%) 457 (100, $[M - H]^-$); LC-TOF-MS *m*/*z* 457.1174 ($[M - H]^-$, measured), *m*/*z* 457.3607 ($[M - H]^-$, calcd for $[C_{16}H_{26}O_{15} - H]^-$); ^1H NMR (400 MHz, D_2O; COSY) *δ* 5.28 [d, 1H, H−C(1′)], 4.07 [d, 1H, H−C(1″)], 3.92 [m, 1H, H_b−C(6′)], 3.91 [m, 1H, H−C(2″)], 3.89 [m, 1H, H_a−C(6′)], 3.88 [m, 1H, H−C(2)], 3.75 [m, 1H, H−C(5′)], 3.74 [m, 1H, H−C(3″)], 3.67 [m, 1H, H−C(4″)], 3.64 [t, 1H, H−C(3′)], 3.58 [m, 2H, H−C(6″)], 3.53 [m, 1H, H−C(4′)], 3.41 [m, 1H, H−C(2′)], 3.33 [m, 1H, H−C(5″)], 2.82 [dd, 1H, H_b−C(3)], 2.72 [dd, 1H, H_a−C(3)]; ^{13}C NMR (400 MHz, D_2O; HMQC, HMBC) *δ* 174.45 [C(4)], 173.15 [C(1)], 103.31 [C(1″)], 92.14 [C(1′)], 81.30 [C(5′)], 76.29 [C(5″)], 73.70 [C(2″)], 70.79 [C(2′)], 71.60 [C(3″)], 71.59 [(C2)], 69.01 [C(3′)], 62.37 [C(6″)], 61.24 [C(6′)], 59.95 [C(4″)], 54.12 [C(4′)], 34.23 [C(3)].

Acidic Hydrolysis of (*S*)-Malic Acid 1′-*O*-*β*-Gentiobioside and Analysis of Carbohydrates. An aliquot (300 *μ*g) of the purified glycoside was taken up in water (300 *μ*L), hydrochloric acid (4.0 mol/L; 300 *μ*L) was added, and, after the mixture had been heated for 30 min at 110 °C, the solution was diluted with water (500 *μ*L) and analyzed for monosaccharides by means of high-performance ion chromatography (HPIC) on an ICS-2500 ion chromatography system (Dionex, Idstein, Germany) equipped with a 250 × 2 mm CarboPac PA-10 column (Dionex), respectively, following the protocol reported earlier.[21]

Synthesis of (*S*)-Malic Acid 1′-*O*-*β*-Glucoside (2). Activated molecular sieve (4 Å; 5 g) was added to a suspension of silver trifluoromethane sulfonate (5.0 mmol) in anhydrous 1,2-dichloroethane (60 mL) under an argon atmosphere in the dark while stirring at room temperature. After cooling to −20 °C, *α*-bromotetra-*O*-acetyl-D-glucose (5.0 mmol) and (*S*)-malic acid dimethyl ester (2.5 mmol) were successively added with stirring. After 10 and 30 min, 2,6-di-*tert*-butyl-4-methylpyridine (5.0 mmol each) was added and, after an overall reaction time of 60 min at −20 °C, the reaction mixture was brought to room temperature and stirred for an additional 12 h. The solution was filtered, the solvent was removed under vacuum, and the oily residue was applied onto the top of a 250 × 30 mm glass column filled with a slurry of silica gel (silica gel 60; 5% water) in toluene. Elution with a toluene/ethyl acetate mixture (2:1, v/v), followed by evaporation of the solvent afforded the dimethyl ester of the target compound as a yellow oil. For deprotection, a solution of the dimethyl ester (1.0 g) in acetone (12.5 mL) was cooled to 0 °C in an ice bath and, after addition of an aqueous calcium hydroxide solution (100 mmol/L, 100 mL), was stirred for 60 min. Thereafter, another aliquot of aqueous calcium hydroxide solution (100 mmol/L, 100 mL) was added, and the mixture was stirred for an additional 3 h at 0 °C, followed by adjustment of the pH value to pH 4 with glacial acetic acid. Addition of anhydrous ethanol (600 mL) and storage at −20 °C overnight afforded the calcium salt of the target compound as a white precipitate, which was separated by membrane filtration (0.45 *μ*m i.d.) and washed with cooled anhydrous ethanol (10 mL). An aliquot of the calcium salt (1.5 mmol) was mixed with a solution of oxalic acid (1.5 mmol) in water (25 mL), the precipitated calcium oxalate was removed by filtration, and the filtrate was lyophilized to obtain (*S*)-malic acid 1′-*O*-*β*-D-glucopyranoside (1.3 mmol) as a white amorphous powder.

*(S)-Malic acid 1′-O-β-D-glucopyranoside (**2**, Figure 4):* LC-MS (ESI^-) *m*/*z* 295 (100); ^1H NMR (500 MHz, D_2O) *δ* 2.88 [dd, 1H, H_a−C(3)], 2.91 [dd, 1H, H_b−C(3)], 3.31 [d, 1H, H−C(2′)], 3.35 [m, 1H, H−C(4′)], 3.36 [m, 1H, H−C(5′)], 3.46 [m, 1H, H−C(3′)], 3.66 [d, 1H, H_a−C(6′)], 3.83 [dd, 1H, H_b−C(6′)], 4.50 [d, 1H, H−C(1′)], 4.57 [d, 1H, H−C(2)]; ^{13}C NMR (125 MHz, D_2O) *δ* 38.2 [C(3)], 60.2 [C(6′)], 69.1 [C(5′)], 73.0 [C(2′)], 75.2 [C(3′)], 75.2 [C(2)], 76.0 [C(4′)], 102.1 [C(1′)], 175.1 [(C(1)], 176.9 [C(4)].

Quantitation of (*S*)-Malic Acid 1′-*O*-*β*-Gentiobioside (1). Samples of freeze-dried vegetables (0.25 g) were extracted with water (3 × 30 mL), and the combined aqueous layers were centrifuged (3500 U/min) for 15 min at 10 °C and then made up with water to 100.0 mL. After membrane filtration, aliquots (20 *μ*L) were analyzed by means of HPLC-MS/MS, and compound **1** was quantitated by means of external standard calibration.

Saliva and Acid Stability of (*S*)-Malic Acid 1′-*O*-*β*-Gentiobioside (1). Aliquots (20 *μ*L) of a solution of **1** (1.0 mg) in water (2 mL) were mixed with hydrochloric acid (140 mmol/L), human pooled saliva (20 *μ*L), or water (20 *μ*L, control), respectively. After incubation for 4 h at 37 °C in the dark, glucoside **1** was quantitated in these samples by means of HILIC-MS/MS.

High-Performance Liquid Chromatography (HPLC). Analytical chromatography was performed on a HPLC apparatus (Jasco, Groß-Umstadt, Germany) equipped with a type PU-2087 PLUS HPLC pump, an AS-2055 PLUS type autoinjector unit, a DAD MD2010 PLUS type detector, and a Sedex 85 type evaporative light scattering detector (LT-ELSD, Sedere S.A., Alfortville Cedex, France), which was operated at 40 °C with air as nebulizer gas (3.5 bar). Data acquisition was done by means of Chrompass software. Analytical separations were performed on a 300 × 7.8 mm i.d., 5 *μ*m, HILIC column (TSKgel Amide-80, Tosoh Bioscience) operated at a flow rate of 1.0 mL/min.

Semipreparative chromatography was performed by HPLC (Gilson, Limburg-Offheim, Germany) equipped with a 322 type pump system and H2 pump heads and a Sedex 85 type evaporative light scattering detector (LT-ELSD, Sedere S.A.), which was operated at 40 °C with air as nebulizer gas (3.5 bar). The chromatographic separation was performed on a 300 × 21.5 mm i.d., 10 *μ*m, TSKgel Amide-80 column (Tosoh Bioscience) operated at a flow rate of 6 mL/min.

High-Performance Liquid Chromatography−Mass Spectrometry (HPLC-MS/MS). Mass spectral analysis was performed in electrospray ionization mode on an API 3200 LC-MS/MS system (AB Sciex Instruments, Darmstadt, Germany) connected to an Agilent 1100 series HPLC system (Agilent, Waldbronn, Germany). The spray voltage was set at −4500 V in the negative electrospray ionization (ESI^-) mode and at 5500 V in the ESI^+ mode. The nebulizer gas was zero-grade air (45 psi), whereas the curtain gas was nitrogen (35 psi). The quadrupoles operated at unit mass resolution. The declustering potential was set at −30 V in the ESI^- mode and at 30 V in the ESI^+

mode used in separate analyses. The mass spectrometer was operated in the full scan mode monitoring positive as well as negative ions. Electrospray ionization (ESI) spectra were acquired with direct loop injection of the samples (2.0−5.0 μL). Fragmentation of $[M - H]^-$ and $[M + H]^+$ pseudomolecular ions into specific product ions was induced by collision with nitrogen (4×10^{-5} Torr) and a collision energy of −20 to −50 V in the ESI^- mode and 20−50 V in the ESI^+ mode. Mass spectrometric data were analyzed by means of Analyst software 1.4.2.

For HPLC-MS/MS analysis, the mass spectrometer was coupled to an Agilent 1100 pump, an Agilent 1100 degasser, and an Agilent 1200 autosampler. The nebulizer gas was zero-grade air (45 psi), whereas the curtain gas was nitrogen (35 psi). For the analysis of **1** by means of the MRM mode, the characteristic mass transition m/z 457 → 133 was recorded after collision-induced dissociation. The declustering potential was set at −10 V, collision energy at −5 V, and cell exit potential at −10 V. The quadrupoles operated at unit mass resolution. For instrumentation control and data collection Sciex Analyst software (v1.4.2) was used. After sample injection (20.0 μL), chromatography was performed on a 300 × 7.8 mm i.d., 5 μm, HILIC column containing carbamoyl-derivatized silica gel (TSKgel Amide-80, Tosoh Bioscience) with gradient elution at a flow rate of 1.0 mL/min. Chromatography was performed by starting with a mixture (5:95, v/v) of 0.1% aqueous formic acid and acetonitrile for 10 min and then increasing the aqueous content to 100% within 20 min.

Analysis of hippuric acid by means of HPLC-MS/MS was performed on a 150 × 2 mm, 5 μm, Synergi Fusion RP-80 column (Phenomenex) equipped with a guard column of the same type. Chromatography was done at a flow rate of 0.25 mL/min using an isocratic solvent mixture (25:75, v/v) of acetonitrile containing 0.1% formic acid and an aqueous formic acid solution (0.1% in water). Using the MRM mode, hippuric acid was detected by recording the characteristic mass transition m/z 177 → 133 after collision-induced dissociation. The declustering potential was set at −25 V, collision energy at −16 V, and cell exit potential at −2 V.

LC/Time-of-Flight Mass Spectrometry (LC/TOF-MS). High-resolution mass spectra of the compounds were measured on a Bruker Micro-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany).

NMR Spectroscopy. NMR data were obtained on Bruker DPX-400 and Bruker 500 MHz Avance III spectrometers (Bruker BioSpin, Rheinstetten, Germany), respectively, using D_2O as the solvent. Chemical shifts were referenced to the solvent signal. For structural elucidation and signal assignment, COSY, HMQC, and HMBC experiments were carried out using the pulse sequences taken from the Bruker software library. Data processing was performed using the software Mestre Nova 5 (Mestrelab Research, Santiago de Compostela, Spain).

RESULTS AND DISCUSSION

To screen a selection of vegetables and spices for their ACE-inhibitory activity, samples of leek, lettuce, parsley, fennel, Swiss chard, celery stalks, garlic, pepper, tomato, onion, and white cabbage were freeze-dried and extracted with methanol/water mixtures. The in vitro ACE-inhibitory activity was then quantitatively determined by HPLC-MS/MS_{MRM} analysis of the ACE-catalyzed release of hippuric acid from the substrate hippuryl L-histidyl-L-leucine when incubated in the presence and absence of the botanical extracts (0.33 mg/mL), respectively.

By far the highest ACE-inhibitory activity of 76.5% was found by the extract prepared from lettuce, followed by white cabbage and Swiss chard extracts inhibiting the ACE activity by 57.7 and 45.7%, respectively (Table 1). In comparison, leek, parsley, and tomato showed only moderate effects between 20.2 and 25.5%, and garlic, pepper, onion, celery stalks, and fennel did not show any inhibitory activity at all. On the basis of the results of this prescreening, the lettuce extract was selected for activity-guided fractionation targeted toward the identification of the key ACE inhibitor.

Table 1. ACE Inhibitory Activity of Vegetable and Spice Extracts

sample[a]	ACE inhibition[b] (%)	sample[a]	ACE inhibition[b] (%)
lettuce	76.5 (±1.4)	pepper	0.8 (±1.7)
leek	25.5 (±1.7)	garlic	−1.7 (±2.7)
white cabbage	57.7 (±1.4)	fennel	−2.6 (±1.8)
Swiss chard	45.7 (±1.4)	onion	−3.5 (±2.8)
parsley	20.4 (±1.9)	celery stalks	−5.3 (±0.6)
tomato	20.2 (±0.7)		

[a]Isolates used were prepared by extraction of botanicals with methanol/water (70:30), followed by freeze-drying. [b]Data are given as the mean of triplicates (relative standard deviation). Final test concentration of each extract was 0.33 mg/mL.

Activity-Guided Discovery of the Key ACE-Inhibiting Principle in Lettuce. To separate the ACE-inhibiting compounds from the bulk of nonactive components, the lettuce extract was resolved by means of medium-pressure column chromatography on RP-18 material using water/acetonitrile mixtures as the mobile phase. Four fractions were collected and freeze-dried to give fractions I−IV as amorphous powders in yields of 88, 6, 4, and 2%, respectively (Table 2).

Table 2. ACE Inhibitory Activity of RP-18 Fractions Isolated from Lettuce

fraction	water/acetonitrile[a] (v/v)	yield (%)	ACE inhibition[b] (%)
I	100/0	88	98.5 (±0.5)
II	95/5	6	41.5 (±0.5)
III	90/10	4	63.5 (±0.6)
IV	50/50	2	69.8 (±0.8)

[a]Water/acetonitrile mixtures used as effluent. [b]Data are given as the mean of triplicates (relative standard deviation).

Dilutions of fractions I−IV in their "natural" concentration ratios, that is, I (1.2 mg/100 mL), II (0.08 mg/100 mL), III (0.05 mg/100 mL), and IV (0.02 mg/100 mL), were analyzed for ACE inhibition. The highest activity was found for the polar fraction I, decreasing the ACE activity by 98.5% (Table 2). Determination of the half-maximum inhibitory concentration (IC_{50}) of this potent fraction I revealed a value of 0.6 mg/100 mL (Figure 1).

To further resolve the highly polar compounds in the active fraction, fraction I was further separated by means of a semipreparative HILIC to give the subfractions I/1−I/6, which were applied in their "natural" concentration in the in vitro ACE inhibition assay (Figure 2). Although all six subfractions showed ACE-inhibitory activity, fractions I/1, I/2, and I/6 were most potent and inhibited ACE activity by 99.5 and 95.7%, respectively. To locate the most active inhibitor, 1:5 dilutions of the solubilized fractions were assayed again. Whereas the diluted fraction I/6 still inhibited ACE activity by 79.3%, the inhibitory power of the diluted fractions I/1 and I/2 was decreased to only 8.5 and 11.1%, respectively (Figure 2). Therefore, the following investigations were targeted toward the identification of the most active phytochemical in fraction I/6.

Rechromatography of fraction I/6 by means of semi-preparative HILIC using a modified solvent gradient yielded

five subfractions, namely, I/6a–I/6e, which were collected individually, freed from solvent under vacuum, taken up in the same amount of buffer solution, and then analyzed for their ACE-inhibitory activity. Although the solutions of fractions I/6c and I/6e were found to inhibit ACE activity to similar extents, that is, 23.2 and 22.0%, the IC_{50} values of both fractions differed strongly; values of 11.7 and 0.01 mg/mL were found for fractions I/6c and I/6e, respectively.

LC-MS analysis of the isolated fraction I/6e revealed a compound (**1**) exhibiting a molecular mass of 458 Da. LC-MS/MS (ESI^-) analysis of the pseudomolecular ion m/z 457 ($[M - H]^-$) revealed the loss of 324 amu to give the fragment ion m/z 133, thus indicating the cleavage of a dihexose moiety. Acidic hydrolysis, followed by high-performance ion chromatographic analysis of carbohydrates, led to the identification of the disaccharide gentiobiose besides somewhat lower amounts of glucose, thus indicating the presence of a gentiobiose moiety in the target compound **1**. This was further strengthened by means of 1D- and 2D-NMR experiments, which allowed the unequivocal identification of compound **1** as (*S*)-malic acid 1′-*O*-*β*-D-gentiobioside. The two geminal protons observed in the ^1H NMR spectrum of **1** could be assigned as H_a–C(3) and H_b–C(3) due to the large coupling constant of 16.9 Hz (Figure 3). 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 3.88 ppm. Heteronuclear multiple-quantum correlation spectroscopy (HMQC) optimized for $^1J_{C,H}$ coupling constants and heteronuclear multiple-bond correlation spectroscopy (HMBC) optimized for $^2J_{C,H}$ and $^3J_{C,H}$ coupling constants revealed a $^2J_{C,H}$ correlation between the methylene protons H–C(3a) and H–C(3b) and the carboxy carbon C(4) resonating at 174.45 ppm as well as between the proton H–C(2) and the second carboxy carbon observed at 173.15 ppm. As expected, the anomeric protons H–C(1′) and H–C(1″) of the gentiobioside moiety resonating at 5.28 and 4.07 ppm, respectively, showed heteronuclear correlation with the carbon resonance signals at 92.14 and 103.31 ppm, respectively. With all of these data taken into account, the ACE inhibitor **1** isolated from fraction I/6e could be unequivocally identified as (*S*)-malic acid 1′-*O*-*β*-D-gentiobioside (Figure 4). To the best of our knowledge, this glycoside has not been previously reported in the literature and presents a new class of phytochemicals with in vitro ACE-inhibitory activity found in a plant food.

ACE-Inhibitory Activity of (*S*)-Malic Acid Glycosides. Prior to the determination of its IC_{50} value for ACE inhibition, the purity of (*S*)-malic acid 1′-*O*-*β*-D-gentiobioside (**1**) was checked to be >98% by means of HILIC-ELSD and ^1H NMR spectroscopy. Using the in vitro assay, glycoside **1** was found to show a rather low IC_{50} value of 27.8 μmol/L (Figure 5A). As the structurally related (*S*)-malic acid 1′-*O*-*β*-D-glucoside (**2**), coined (*S*)-morelid, was recently identified in morel mushrooms,[25] this glucoside was synthesized, purified, and then used for the analysis of its ACE-inhibitory activity. Compared to gentiobioside **1**, the corresponding glucoside **2** showed a ∼300 times higher IC_{50} value of 7800 μmol/L (Figure 5B), thus demonstrating the strong influence of the carbohydrate moiety on the ACE-inhibitory activity. To validate the specificity of the ACE-inhibitory activity found for glycosides **1** and **2**, the test assay was performed with their single components malic acid, gentiobiose, and glucose, respectively. However, none of these compounds showed any significant ACE-inhibitory activity, thus confirming the prime importance of the glycosidic bond as

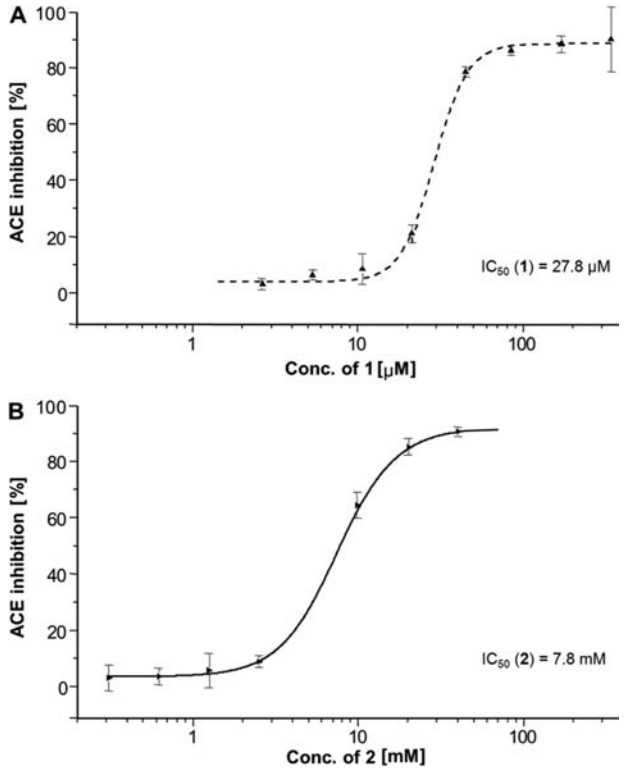

Figure 5. Dose–response curves for ACE inhibition of (A) (*S*)-malic acid 1′-*O*-*β*-D-gentiobioside (**1**) and (B) (*S*)-malic acid 1′-*O*-*β*-D-glucoside (**2**).

proposed earlier for synthetic glycosides such as eugenyl *O*-*β*-D-glucoside and vanillyl *O*-*β*-D-mannoside, respectively.[26]

Concentrations of (*S*)-Malic Acid 1′-*O*-*β*-D-Gentiobioside in Lettuce. To determine the contribution of (*S*)-malic acid 1′-*O*-*β*-D-gentiobioside (**1**) to the ACE-inhibitory activity of lettuce, glycoside **1** was quantitated in aqueous extracts made from *L. sativa* varieties by means of HILIC-MS/MS(ESI^-) operating in the MRM mode and recording the characteristic mass transition m/z 457 → 133 after collision-induced dissociation.

The concentration of **1** in the seven lettuce varieties investigated ranged from 12.9 to 170.8 mg/100 g fresh weight (Table 3). Iceberg lettuce and butterhead lettuce, both belonging to the variety *capitata*, contained the target glycoside in high concentrations of 170.8 and 137.9 mg/100 g (fresh weight), respectively. In comparison, green oak leaf lettuce, red oak leaf lettuce, lollo rosso, and lollo bionda, all of which

Table 3. Concentration of (*S*)-Malic Acid 1′-*O*-*β*-D-Gentiobioside (1) in Lettuce Varieties

sample	variety	concn[a] (mg/100 g)	concn/IC_{50}[b]
iceberg lettuce	*capitata*	170.8 (±7.6)	134.5
butterhead lettuce	*capitata*	137.9 (±0.7)	108.6
romaine lettuce	*longifolia*	115.3 (±1.2)	90.8
green oak leaf lettuce	*crispa*	30.7 (±1.3)	24.2
red oak leaf lettuce	*crispa*	29.0 (±1.7)	22.8
lollo rosso	*crispa*	15.1 (±0.1)	11.9
lollo bionda	*crispa*	12.9 (±0.3)	10.2

[a]Concentrations are based on fresh weight and are given as the mean of triplicates (relative standard deviation). [b]Ratio of concentration and IC_{50} value (1.27 mg/100g) of **1**.

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belong to the *crispa* variety, contained compound **1** at considerably lower levels of 12.9−30.7 mg/100 g. It is interesting to note that the concentrations of **1** in the lettuce samples were between 11.9 and 134.5 times above the IC_{50} value (1.27 mg/100 g) determined for the purified glycoside, thus showing evidence for (*S*)-malic acid 1′-*O*-*β*-D-gentiobioside to be a major contributor to the ACE-inhibitory effect of lettuce. This suggestion was further supported by the good correlation found between the ACE-inhibitory activity of the aqueous lettuce extracts and the concentrations of **1** determined by means of HILIC-MS/MS (Figure 6). Additional

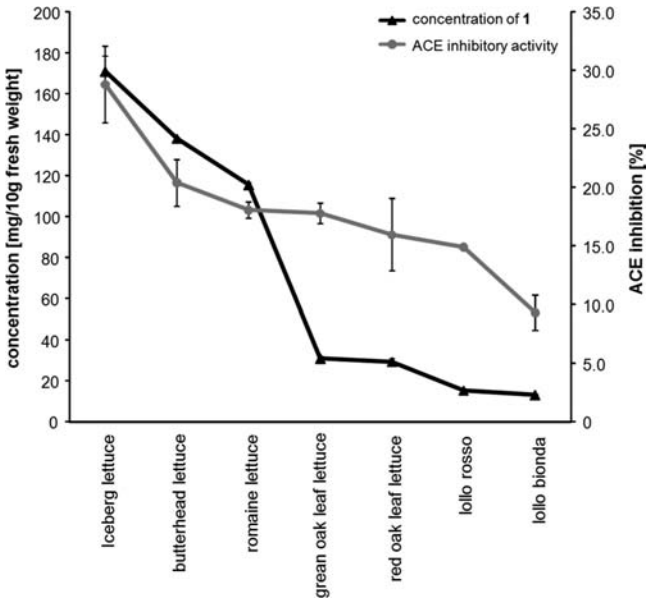

Figure 6. Correlation of the concentration of (*S*)-malic acid 1′-*β*-gentiobioside (**1**) and the ACE-inhibitiory activity of lettuce varieties.

HILIC-MS/MS analysis of (*S*)-morelid (**2**) in the lettuce sample revealed only rather low amounts below 1.0 mg/100 g (data not shown). In consideration of the high IC_{50} value of 2.3 mg/mL, this glucoside can be excluded as an ACE inhibitor.

Saliva and Acid Stability of (*S*)-Malic Acid 1′-*O*-*β*-Gentiobioside (1). To gain some first insight into the stability of glycoside **1** in the presence of salivary enzymes and stomach acid, aqueous solutions of **1** were incubated for 4 h at 37 °C in human pooled saliva, in aqueous hydrochloric acid (140 μmol/L), and in water (control), respectively, and the remaining amount of the glycoside was analyzed by means of HILIC-MS/MS. When compared to the control, incubation with saliva and hydrochloric acid revealed recoveries of 91.3 and 60.0%, respectively, of the intact glycoside **1**, thus demonstrating a rather high stability of the glycoside under conditions mimicking stomach passage.

In conclusion, activity-guided fractionation of lettuce using an in vitro assay, followed by LC-MS and NMR experiments, as well as quantitative studies led to the unequivocal identification of (*S*)-malic acid 1′-*O*-*β*-D-gentiobioside (**1**) as the major contributor to the ACE-inhibitory effect of lettuce. First incubation experiments in saliva and aqueous hydrochloric acid demonstrated the potential stability of the target glycoside after ingestion, but bioavailability, plasma half-life time, and blood pressure measurements are needed in future studies to evaluate the in vivo ACE-inhibitory activity of this novel glycoside.

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Notes

The authors declare no competing financial interest.

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