Isolation and Characterization of Structurally Novel Antimutagenic Flavonoids from Spinach (*Spinacia oleracea*)

Rudolf Edenharder,*,† Gernot Keller,‡ Karl L. Platt,§ and Klaus K. Unger‡

Department of Hygiene and Environmental Medicine, University of Mainz, Obere Zahlbacher Strasse 67, D-55131 Mainz, Germany; Institute of Toxicology, University of Mainz, Obere Zahlbacher Strasse 67, D-55131 Mainz, Germany; and Institute of Inorganic Chemistry and Analytical Chemistry, University of Mainz, Duesbergweg 10-14, D-55099 Mainz, Germany

Thirteen compounds, isolated from spinach (*Spinacia oleracea*), acted as antimutagens against the dietary carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline in *Salmonella typhimurium* TA 98. The antimutagens were purified by preparative and micropreparative HPLC from a methanol/water (70:30, v/v) extract of dry spinach (commercial product) after removal of lipophilic compounds such as chlorophylls and carotenoids by solid-phase extraction (SPE). Pure active compounds were identified by instrumental analysis including FT-IR, ^{1}H and ^{13}C NMR, UV−vis spectroscopy, and mass spectrometry. All of these compounds were flavonoids and related compounds that could be attributed to five groups: (A, methylenedioxyflavonol glucuronides) 5,3′-dihydroxy-4′-methoxy-6,7-methylenedioxyflavonol 3-*O*-*β*-glucuronide (compound **1**), 5,2′,3′-trihydroxy-4′-methoxy-6,7-methylenedioxyflavonol 3-*O*-*β*-glucuronide (compound **2**), 5-hydroxy-3′,4′-dimethoxy-6,7-methylenedioxyflavonol 3-*O*-*β*-glucuronide (compound **3**); (B, flavonol glucuronides) 5,6,3′-trihydroxy-7,4′-dimethoxyflavonol 3-*O*-*β*-glucuronide (compound **4**), 5,6-dihydroxy-7,3′,4′-trimethoxyflavonol 3-*O*-*β*-glucuronide (compound **5**); (C, flavonol disaccharides) 5,6,4′-trihydroxy-7,3′-dimethoxyflavonol 3-*O*-disaccharide (compound **6**), 5,6,3′,4′-tetrahydroxy-7-methoxyflavonol 3-*O*-disaccharide (compounds **7** and **8**); (D, flavanones) 5,8,4′-trihydroxyflavanone (compound **9**), 7,8,4′-trihydroxyflavanone (compound **10**); (E, flavonoid-related compounds) compounds **11**, **12**, and **13** with incompletely elucidated structures. The yield of compound **1** was 0.3%, related to dry weight, whereas the yields of compounds **2**−**13** ranged between 0.017 and 0.069%. IC_{50} values (antimutagenic potencies) of the flavonol glucuronides ranged between 24.2 and 58.2 μM, whereas the flavonol disaccharides (compounds **7** and **8**), the flavanones (compounds **9** and **10**), and the flavonoid-related glycosidic compounds **11**−**13** were only weakly active. The aglycons of compounds **7** and **8**, however, were potent antimutagens (IC_{50} = 10.4 and 13.0 μM, respectively).

Keywords: *Spinach; 2-amino-3-methylimidazo[4,5-f]quinoline; flavonoids; antimutagenic activity; Salmonella/reversion assay*

INTRODUCTION

In more than 200 case-control and several human cohort studies it was shown that a high consumption of fruits and vegetables is consistently associated with a low incidence for all common cancer sites except for hormone-dependent breast and prostate cancers (*1*−*4*). On the whole, epidemiological evidence is now considered overwhelming (*5*), and particularly strong associations were detected for cancers of the alimentary and respiratory tracts. Associations exist for a wide variety of fruits and vegetables, although available evidence suggests some differences for specific sites (*1*). In model experiments with rodents, protective effects of common vegetables of the human diet, mostly cruciferous ones, against the induction of cancer have been found, confirming the epidemiological evidence (*6*). Similar results have also been obtained in less expensive short-term assays: In the in vivo mouse bone-marrow micronucleus assay various fruits and vegetables reduced clastogenic activities of model compounds (*7*, *8*), and in the in vitro *Salmonella*/reversion assay a great number of fruits and vegetables exerted protective effects against mutagenicity induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline, other heterocyclic amines from cooked food, and polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene (*9*−*12*). These compounds are carcinogenic in laboratory animals and possibly in humans, too. In our investigations particularly strong and consistent anticlastogenic and antimutagenic effects of spinach against 2-amino-3-methylimidazo[4,5-*f*]quinoline were seen, although the relevant active compounds were largely unknown. The objective of this study was to identify compounds with antimutagenic potential against this imidazoquinoline in the *Salmonella*/reversion assay among the multitude of compounds present in spinach by activity-directed enrichment, followed by purification and spectroscopic identification.

* Corresponding author (telephone + 49-3161-3933163; fax + 49-3163-3936628; e-mail edenhard@uni-mainz.de).
† Department of Hygiene and Environmental Medicine.
‡ Institute of Chemistry and Analytical Chemistry.
§ Institute of Toxicology.

MATERIALS AND METHODS

Materials. Dry powders of spinach (*Spinacia oleracea*) were obtained from Bestfoods Europe, Heilbronn, Germany. The mutagen 2-amino-3-methylimidazo[4,5-*f*]quinoline was pur-

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chased from Toronto Research Chemicals, Downsview, ON, Canada. Reference compounds such as chlorophyll, carotenoids, and flavonoids were obtained from Roth, Karlsruhe, Germany, or Fluka, Neu-Ulm, Germany; sugar standards as well as galacturonic and glucuronic acids were from Aldrich, Steinheim, Germany. Glucose-6-phosphate and NADP were purchased from Boehringer (Roche), Mannheim, Germany. All solvents including dimethyl sulfoxide (DMSO) as well as chemicals not specifically indicated were from Merck, Darmstadt, Germany. Solvents were of the highest grade necessary, pro analysi, chromatographically pure or gradient quality.

Extraction and Isolation. The dry spinach powder (100 g) was extracted twice with 10 volumes of methanol/water (70:30, v/v) at 40 °C for 2 h by stirring, and the extracts were filtered and combined. By this procedure, a maximum of materials (~5.5 g/100 g) could be extracted with maximum amounts of flavonoids present (45% flavonoids, 10% chlorophylls, 10% epiphasic carotenoids, 35% hypophasic carotenoids). With other solvents and solvent mixtures such as ethanol/water (50:50, v/v), 2-propanol/water (50:50, v/v), methanol, ethanol, 2-propanol, and *n*-hexane the contributions of flavonoids were 35, 35, 35, 10, 10, and 0%, respectively, whereas the amounts of chlorophylls and carotenoids increased appropriately. Soxhlet extraction was not superior to the batch procedure, neither on a mass basis nor when related to antimutagenic activities. Application of the solvents hexane, dichloromethane, acetone, and 2-propanol in the sequence of polarity as performed in previous investigations with plant residues (*12*) resulted in 40−70% overlapping of selectivity. To remove chlorophylls and carotenoids, up to 30 g of a reversed phase packing, ODS-C_{18} (Baker, Gross-Gerau), was added to the aqueous/methanolic spinach extract, shaken for 30 min, and then centrifuged. The solid material was washed twice with 2 volumes of methanol/water (70:30) and the washings were combined with the original extract. This solution was concentrated under reduced pressure to remove methanol and a part of the water, then frozen at −20 °C, and freeze-dried to protect potentially sensitive compounds.

Evaluation of Antimutagenicity against 2-Amino-3-methylimidazo[4,5-*f*]quinoline and Related Procedures. The antimutagenicity of extracts, HPLC fractions, and compounds was evaluated as reported previously (*11*−*13*). The following components were added in order: 500 μL of isotonic KCl, 20 ng of mutagen (2353 ± 411 revertants/plate), dissolved in 50 μL of dimethyl sulfoxide, 200 μL of solution of test compound in dimethyl sulfoxide, 500 μL of S9 mix, 100 μL of bacterial suspension [the original broth was centrifuged, the pellet was resuspended in isotonic KCl, and adjusted to an optical density of ~1.0 ± 0.05 at 578 nm (l = 0.5 cm), equivalent to ~2.8×10^9 viable cells/mL], and 2.5 mL of top agar, total volume = 3.85 mL. Procedures were essentially as described by Maron and Ames (*14*) and also for preparation of mammalian activation system (S9 mix) and mutagenicity testing. Toxicity was determined according to the method of Waleh et al. (*15*); the surviving fraction of bacteria was always >0.8 in measuring ranges. No measurements were performed in toxic ranges. Counting of colonies on plates for the *Salmonella*/reversion assay was performed by a Biotran II, automated colony counter (New Brunswick Scientific Co.).

Identification of Antimutagenic Compounds. Antimutagenic compounds, purified by preparative and repeated micropreparative HPLC from spinach, were controlled for purity by analytical HPLC. Pure compounds were identified by instrumental analyses.

Preparative, Micropreparative, and Analytical HPLC. *Preparative HPLC.* Thirty-six milliliters of solutions (possible solvents: water, methanol, water/methanol mixtures, 2-propanol, dichloromethane, and hexane), concentration up to 40 mg/mL, was applied (6 mL sample loop, six times) to a preparative YMC ODS-AQ column (50 mm × 260 mm, 10 μm particle size, Prochrom, Champigneulles, France). Gradient elution was carried out stepwise with water and methanol, each solvent containing 0.01% trifluoroacetic acid (TFA). Actual elution conditions were as follows: water for 0−5 min, water/methanol (80:20, v/v) for 5−12 min, water/methanol (70:30, v/v) for 12−20 min, water/methanol (60:40, v/v) for 20−40 min, water/methanol (40:60, v/v) for 40−45 min, and water/methanol (20:80, v/v) at ambient temperature and at a flow rate of 60−80 mL/min. The eluate was monitored with a photodiode array detector (DAD) at a wavelength of 315 nm. Fractions were collected according to the elution of peaks (see Figure 1), eventually concentrated, and further processed by micropreparative procedures.

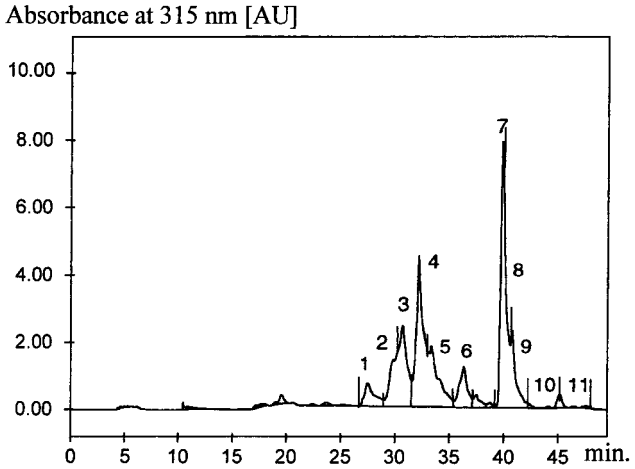

Figure 1. Preparative HPLC chromatogram of the methanol/water extract (70:30, v/v) from dry spinach (commercial product) after removal of lipophilic compounds by SPE. For a detailed description of the procedure, see Materials and Methods.

Micropreparative HPLC. Two hundred microliters of fractions, obtained by preparative HPLC, was applied (100 μL sample loop, two times) to a YMC ODS-AQ column (5 μm particle size, 4.6 mm × 250 mm). Gradient elutions were again carried out with methanol and water, containing 0.01% TFA, at ambient temperature at a flow rate of 1 mL/min; eluates were monitored by DAD at 315 nm. Elution conditions were as follows: micropreparative I, fractions 1 and 3 (Figure 1), methanol/water (75% water) for 0−2 min, methanol/water (75−70%) for 2−7 min, methanol/water (70%) for 7−8 min, methanol/water (70−60%) for 8−10 min, methanol/water (60%) for 10−12 min; micropreparative II, fractions 5 and 6, methanol/water (70% water) for 0−2 min, methanol/water (70−60%) for 2−5 min, methanol/water (60%) for 5−9 min; micropreparative III, fractions 8 and 9, methanol/water (65−55% water) for 0−7 min; micropreparative IV, fractions 10 and 11, methanol/water (50% water) for 0−3 min, methanol/water (50−40%) for 3−6 min, methanol/water (40%) for 6−8 min, methanol/water (40−20%) for 8−12 min. Fractions 2 and 4 of Figure 1 were not antimutagenic and were therefore not analyzed further; fraction 7 contained the pure compound **1**. It was necessary to perform up to 20 runs to obtain sufficient amounts (5−10 mg) of compounds to be characterized by antimutagenic potency and structure. Again, fractions were collected according to the elution of peaks and were analyzed for purity by analytical HPLC.

Analytical HPLC. An amount of 10−20 μL of a solution with flavonoids (glycosides, aglycons) or related compounds, dissolved in water, methanol/water, methanol, 2-propanol, or dichloromethane, was applied to a YMC ODS-AQ column (4.6 mm × 250 mm, 5 μm particle size) or a Bischoff ODS column (2.6 mm × 250 mm, 3 μm particle size). Solvents for elution were water and acetonitrile, both containing 0.01% TFA. Actual elution conditions were (analytical II) as follows: water for 0−10 min, gradient elution with 100−50% water for 10−40 min and 50−0% water for 40−50 min at a temperature of 30 °C and at a flow rate of 1 or 0.35 mL/min (second column). The eluate was monitored with a DAD at 260 and 315 nm. In later experiments, the isocratic elution phase could frequently be eliminated due to sufficient separation efficiency (analytical IIa). Other variations were as follows: analytical VI for methylated flavonoid aglycons, YMC ODS-AQ column (4.6 mm

× 250 mm, 5 μm particle size), elution gradient 100−50% water for 0−30 min and 50−0% water for 30−40 min, detection at 360 nm. All other conditions were as described under analytical II.

Characterization by Coupled Instrumental Procedures. For liquid chromatography−mass spectrometry (LC-MS) a YMC ODS-AQ microbore column (1 mm × 150 mm, 5 μm particle size) was utilized at ambient temperature and at a flow rate of 0.1 mL/min. Fractions from preparative HPLC were applied, and the conditions were as described under Micropreparative HPLC. UV−vis spectra were recorded, followed by mass spectra. However, mass spectra of only part of the compounds could be obtained because many flavonoid aglycons were eliminated in the particle beam interface. A thermospray interface with chemical ionization was, however, not available. For gas chromatography−mass spectrometry (GC-MS) flavonoid glycosides had to be hydrolyzed and aglycons had to be methylated (see below). Conditions of GC were as follows: 2 μL of the sample was injected to a silica capillary column, length = 15 m, diameter = 0.75 mm, with fused methylsiloxane, film diameter = 0.25 μm, gas (He) flow rate = 2 mL/min, temperature gradient from 100 to 280 °C, (0−2 min), 100 °C, (2−10 min), increase of 10 °C/min, (10−15 min) 180 °C, (15−25 min) an increase of 10 °C/min, and (25−30 min), 280 °C.

Hydrolysis of Glycosides and Methylation of Aglycons. For hydrolysis 5−10 mg of materials from fractions I, II, and IV was dissolved in 5 mL of water, and 0.4 mL of TFA was added. The mixture was refluxed for 60 min at 100 °C. After cooling, a brown precipitate was obtained, which was filtered and dried. Purity of compounds was checked by analytical HPLC procedure VI. Under these experimental conditions only flavonoid aglycons were present in all fractions. For methylation, flavonoids dissolved in acetone were treated with an ether solution of diazomethane until no more nitrogen was generated.

Ion Chromatography with a Pulsed Amperometric Detector (IC-PAD). Glycosides were detected electrochemically by applying a gold electrode. Conditions were as follows: measuring potential, 0.05 V; purification potentials, 0.60 and − 0.60 V; measuring time, 480 ms; purification times, 120 and 60 ms; range, 10 μA; display, positive; response time, 1 s. For chromatography of glycosides 20 μL of a solution was applied to a CarboPac PA 1 column (mono- and disaccharides) or to a CarboPac PA 100 column (oligosaccharides). Glycosides were eluted under isocratic conditions for 20 min with a mixture of 10 mM NaOH and 200 mM NaOH solution (3:1, v/v) at ambient temperature and at a flow rate of 0.8 mL/min. Identification of sugars was based on comparison of retention times with those of respective standards.

Instrumental Analyses. The compounds isolated by chromatography were analyzed by using the following procedures: The Fourier transform infrared (FT-IR) spectra (2 mg/250 mg of KBr) were determined with a Mattson Galaxy 2000 spectrometer and the UV−vis spectra with a UV-240 spectrometer (Shimadzu, Kyoto, Japan). Analytical and micropreparative HPLC was performed with a Hewlett-Packard liquid chromatograph (Agilent Technologies, Waldbronn, Germany). For preparative HPLC two high-pressure pumps (HD 2-200; Besta, Wilhelmsfeld, Germany) and a UV detector (Lambda 1000; Bischoff, Leonberg, Germany) were used. For LC-MS a Waters integrity system (Waters, Milford, MA) with a particle beam interface was used. For gas chromatography−mass spectrometry (GC-MS) a Varian GC 3400 (Varian, Walnut Creek, CA) and a Magnum ITD (Finnigan, Bremen, Germany) were utilized. Electron impact mass spectra (EI-MS) as well as fast atom bombardment mass spectra (FAB-MS) were obtained on a Finnigan-Mat 8200. For FAB-MS compounds were dissolved in DMSO, whereas glycerol was used as the matrix. 1D proton nuclear magnetic resonance (^{1}H NMR) and carbon nuclear resonance (^{13}C NMR) spectra as well as 2D ^{1}H,^{1}H-COSY, and ^{1}H,^{13}C-COSY spectra were recorded on a Bruker AM 250 spectrometer. ^{1}H NMR spectra were performed of all compounds isolated (sample size = 50 μg−2 mg) and of various fractions containing a maximum of three compounds. A ^{13}C NMR spectrum could be recorded only with compound **1** due to the amounts necessary (20−30 mg). All work was performed in a glovebox under an atmosphere of argon because most isolated compounds were hygroscopic. Samples were dissolved in hexadeuteriodimethyl sulfoxide (DMSO-d_6) and were later recovered by solid-phase extraction (SPE) using ODS-reversed phase material, 50 μm (Bakerbond), equilibrated with water (elution of flavonoids with methanol). For analyses of glycosides a DX-300 (Dionex, Idstein, Germany) was used.

RESULTS

Purification of the Antimutagens in Spinach. Extraction of a commercial product of dry spinach (100 g) with the solvent sequence *n*-hexane, dichloromethane, 2-propanol, and methanol/water (50:50, v/v) resulted in the isolation of 0.37, 0.65, 1.2, and 5.3 g of materials, respectively (total yield = 7.52 g). The juice as well as the water insoluble compounds extracted from the residues in these and in preceding investigations exerted strong antimutagenicity against 2-amino-3-methylimidazo[4,5-*f*]quinoline (*11*, *12*). It was already known that besides chlorophyll, spinach probably contains many antimutagens including acidic, basic, and neutral compounds (*12*), so in the present study we analyzed only materials extracted with methanol/water (70:30, v/v) from 108 g of dry spinach in order to identify polar antimutagens. The dried residue of this extract, freed from nonpolar compounds such as chlorophyll(s) and carotenoids by SPE, was dissolved in water and subjected to preparative HPLC. The results are shown in Figure 1.

Identification of the Isolated Antimutagenic Compounds. When fraction 7 of preparative HPLC was subjected to micropreparative HPLC according to procedure III, a single antimutagenic compound with a purity of >95%, designated compound **1**, was detected. Application of the same procedure to the combined fractions 8 and 9 resulted in the separation of compounds **1**, **2**, and **3** with retention times of 7.46, 8.24, and 8.64 min, respectively. Analytical HPLC (procedure IIa) proved that these peaks comprised pure compounds with retention times of 26.0, 27.0, and 27.4 min.

The FT-IR spectrum of compound **1** in KBr showed the presence of an aromatic keto group (ν_{max} = 1550−1650 cm^{-1}), a COOH group (ν_{max} = 1700 cm^{-1}), and at least one phenolic OH function (ν_{max} = 3000 cm^{-1}). An additional broad absorption band at 3600 cm^{-1} could be related to OH vibrations with a typical line broadening due to intra- and intermolecular hydrogen bonding. The EI/MS spectrum showed a base peak at *m*/*z* 344 (A^+, molecular ion of the aglycon $[C_{17}H_{12}O_8]^+$, see Figure 2) and major fragment ion peaks at *m*/*z* 343 ($A^+ - 1$), 301, and 180 as well as minor ion peaks at *m*/*z* 314, 280, 271, and 207. A molecular ion peak of the flavonol glucuronide could not be observed in accordance with the expectation that a cleavage of the O-glycosidic bond occurred, whereas a C-glycosidic bond would have survived. The absence of any signal at M^+ is therefore indicative of an *O*-glucuronide. However, the glucuronic acid has a molecular mass of 177 and a corresponding weak signal could be detected. The ^{13}C NMR spectrum showed signals (see Figure 2 for numbering) at δ (ppm) 151.6 (C-2), 129.0 (C-3), 179.2 (C-4), 153.8 (C-5), 146.8 (C-6), 147.6 (C-7), 89.9 (C-8), 155.1 (C-9), 107.6 (C-10), 123.8 (C-1′), 117.6 (C-2′), 140.3 (C-3′), 138.8 (C-4′), 116.5 (C-5′), 119.7 (C-6′), 75.8 (1″), 72.9 (2″), 75.8 (3″), 71.3

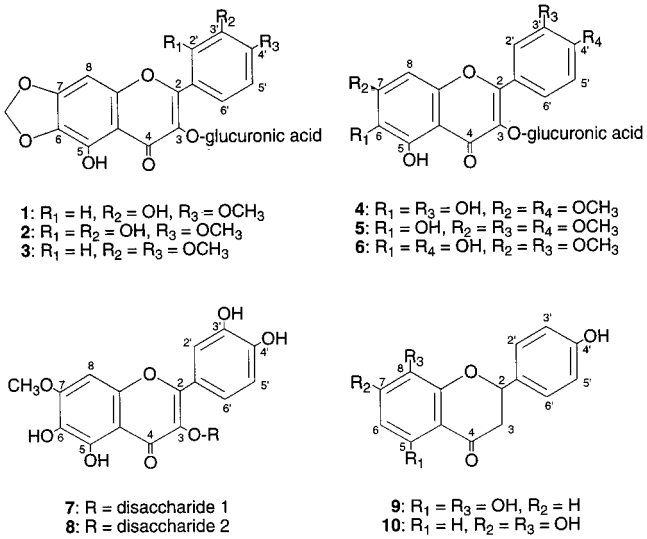

Figure 2. Chemical structures of antimutagenic flavonoids isolated from spinach.

(4″), 75.8 (5″), 170.3 (6″), 138.8 (OCH_3), and 102.6 (OCH_2O). This established the presence of 12 quaternary, 9 tertiary, 1 secondary, and 1 primary carbon atom. The ^{1}H NMR spectrum (DMSO-d_6) gave signals at δ 6.90 (1H, s, 8-H), 7.53 (1H, d, ^4J = 2.5 Hz, 2′-H), 7.17 (1H, d, ^3J = 9.5 Hz, 5′-H), 7.48 (1H, q, ^3J = 9.5 Hz, ^4J = 2.5 Hz, 6′-H), 12.70 (1H, s, 5-OH), 9.2 (1H, s, 3′-OH), 3.80 (3H, s, 4′-OCH_3), 6.15 (2H, s, $-OCH_2O$), 5.00 (1H, d, ^3J = 7.5 Hz, 1″), 3.90 (1H, d, ^3J = 9.5 Hz, 5″), 3.30−3.50 (other sugar protons). The coupling constant of the proton at 1″ indicated a β-linkage (β-anomer, J = 7−8 Hz; α-anomer, J < 7 Hz). The identity of the glycosidic acid could be determined from the NMR data because only galacturonic or glucuronic acid could be expected. Galacturonic acid possesses an equatorially linked H atom at C-4, whereas glucuronic acid possesses an axially linked proton. Both acids possess an equatorially linked H atom at C-5. The vicinal coupling J_{aa} shows a value of >7 Hz. Because the H at G-5 of ^{1}H NMR indicated J = 9.5 Hz, the galacturonic acid could be excluded. During chromatography in acetonitrile/water [0.01% TFA; 50:50 (v/v)] compound **1** showed absorption bands with λ_{max} of 209, 253, 280, and 350 nm, which are typical for flavonoids. Under IC-PAD conditions a peak with a retention time (t_R) of 2.66 min was observed, identical with the reference compound glucuronic acid. These data agree with the assumption that compound **1** in spinach was identical with 5,3′-dihydroxy-4′-methoxy-6,7-methylenedioxyflavonol 3-*O*-β-glucuronide (Figure 2).

On the basis of FT-IR, UV−vis, and ^{1}H NMR spectroscopy, compound **2** in spinach was similarly identified as 5,2′,3′-trihydroxy-4′-methoxy-6,7-methylenedioxyflavonol 3-*O*-β-glucuronide (Figure 2). The FT-IR spectrum, which was very similar to that of compound **1**, indicated the presence of an aromatic carbonyl function, a carboxylic acid group, and phenolic OH group(s). The ^{1}H NMR spectrum showed signals at δ 6.95 (1H, s, 8-H), 7.20 (1H, d, ^3J = 9.5 Hz, 5′-H), 8.02 (1H, d, ^3J = 9.5 Hz, 6′-H), 12.70 (1H, s, 5-OH), 9.20 (2H, s, 2′-OH and 3′-OH), 3.80 (3H, s, 4′-OCH_3), 6.15 (2H, s, OCH_2O), 5.15 (1H, d, ^3J = 7.5 Hz, 1″), 3.90 (1H, d, ^3J = 9.5 Hz, 5″), 3.30−3.50 (other sugar protons). Compound **2** showed UV−vis absorption bands with λ_{max} of 219, 278, and 335 nm.

In the same way, compound **3** in spinach was identified as 5-hydroxy-3′,4′-dimethoxy-6,7-methylenedioxyflavonol 3-*O*-β-glucuronide (Figure 2). Again, the FT-IR spectrum was closely related to that of compound **1**. The EI/MS spectrum gave a base peak at m/z 358 (A^+), major fragment ion peaks at m/z 343 ($A - CH_3$) and 315, and minor signals at m/z 327 and 280. ^{1}H NMR showed signals at δ 6.98 (1H, s, 8-H), 7.65 (1H, d, ^4J = 2.5 Hz, 2′-H), 7.35 (1H, d, ^3J = 9.5 Hz, 5′-H), 7.64 (1H, q, ^3J = 9.5 Hz, ^4J = 2.5 Hz, 6′-H), 12.60 (1H, s, 5-OH), 3.70 (3H, s, 3′-OCH_3), 3.80 (3H, s, 4′-OCH_3), 6.15 (2H, s, OCH_2O-), 5.25 (1H, d, ^3J = 7.5 Hz, 1″), 3.90 (1H, d, ^3J = 9.5 Hz, 5″), 3.30−3.50 (other sugar protons). During chromatography compound **3** showed absorption bands with λ_{max} of 216, 250, 277, and 340 nm, typical for flavonoids.

Fraction 6 of preparative HPLC (see Figure 1) was subsequently subjected to micropreparative HPLC according to procedure II. A series of peaks was detected, among them two major peaks with t_R of 7.37 and 8.00 min, which comprised compounds with antimutagenic activity against 2-amino-3-methylimidazo[4,5-*f*]quinoline. Analytical HPLC, procedure VI, proved that these peaks comprised pure compounds with t_R of 13.66 and 14.75 min, designated compounds **4** and **5**.

After instrumental analysis, compound **4** from spinach was assigned to be 5,6,3′-trihydroxy-7,4′-dimethoxyflavonol 3-*O*-β-glucuronide (Figure 2). The EI/MS spectrum of compound **4** gave a base peak at m/z 346 (A^+), major fragment ions with m/z 345, 331, 328, and 303, and minor signals at m/z 289, 273, 260, 232, 207, 194, 177 (M^+ of glucuronic acid), and 164. The ^{1}H NMR spectrum showed signals at δ 6.51 (1H, s, 8-H), 7.51 (1H, d, ^4J = 2.5 Hz, 2′-H), 7.15 (1H, d, ^3J = 9.5 Hz, 5′-H), 7.47 (1H, q, ^3J = 9.5 Hz, ^4J = 2.5 Hz, 6′-H), 12.60 (1H, s, 5-OH), 9.20 (1H, s, 3′-OH), 3.80 (3H, s, 4′-OCH_3), 10.80 (1H, s, 6-OH), 3.75 (3H, s, 7-OCH_3), 5.05 (1H, d, ^3J = 7.5 Hz, 1″), 3.90 (1H, d, ^3J = 9.5 Hz, 5″), 3.30−3.50 (other protons of glucuronic acid). Compound **4** showed absorption bands with λ_{max} of 208, 252, 270, and 340 nm, again characteristic for flavonoids.

Again, compound **5** from spinach was identified as 5,6-dihydroxy-7,3′,4′-trimethoxyflavonol 3-*O*-β-glucuronide (Figure 2). The FT-IR spectrum indicated the presence of an aromatic carbonyl function, a carboxylic acid group, and a phenolic OH group. The ^{1}H NMR spectrum showed signals at δ 6.56 (1H, s, 8-H), 7.62 (1H, d, ^4J = 2.5 Hz, 2′-H), 7.25 (1H, d, ^3J = 9.5 Hz, 5′-H), 7.58 (1H, q, ^3J = 9.5 Hz, ^4J = 2.5 Hz, 6′-H), 12.60 (1H, s, 5-OH), 3.85 (3H, s, 3′-OCH_3), 3.80 (3H, s, 4′-OCH_3), 10.80 (1H, s, 6-OH), 3.75 (3H, s, 7-OCH_3), 5.05 (1H, d, ^3J = 7.5 Hz, 1″), 3.90 (1H, d, ^3J = 9.5 Hz, 5″), 3.30−3.50 (other protons of glucuronic acid). Compound **5** showed absorption bands with λ_{max} of 208, 253, 270, and 340 nm, nearly identical with those of the spectrum of compound **4**.

When fraction 1 (Figure 1) obtained by preparative HPLC was subjected to micropreparative HPLC, according to procedure I, four major peaks representing compounds with antimutagenic activities against 2-amino-3-methylimidazo[4,5-*f*]quinoline were observed, with t_R values of 3.98 min (compound **7**), 4.97 min (compounds **6** and **8**), 6.36 min (compound **9**), and 6.89 min (compound **10**). Under the analytical HPLC conditions, procedure II, t_R values of 17.15, 17.99, and 18.23 min were observed for the pure compounds **7**, **8**, and **6**, respectively. After instrumental analysis, compound **6**

was identified as a 5,6,4′-trihydroxy-7,3′-dimethoxyflavonol 3-*O*-disaccharide (Figure 2). The ^{1}H NMR spectrum (in DMSO-d_6) indicated signals at δ 6.55 (1H, s, 8-H), 7.90 (1H, d, ^4J = 2.5 Hz, 2′-H), 6.90 (1H, d, ^3J = 9.5 Hz, 5′-H), 7.46 (1H, q, ^3J = 9.5 Hz, ^4J = 2.5 Hz, 6′-H), 12.70 (1H, s, 5-OH), 3.85 (3H, s, 3′-OCH$_3$), 9.80 (1H, s, 4′-OH), 10.75 (1H, s, 6-OH), 3.75 (3H, s, 7-OCH$_3$), 5.50 (1H, d, ^3J = 7.5 Hz, 1″), 4.10 (1H, d, ^3J = 9.5 Hz, 2″), 3.30−3.50 (other sugar protons). During chromatography in acetonitrile/water [0.01% TFA; 50:50 (v/v)] compound **6** showed absorption bands with λ_{max} of 205, 255, 269, and 351 nm, again typical for a flavonoid. When compound **6** was hydrolyzed and the sugars were analyzed by IC-PAD, three peaks were observed: a major peak with t_R of 5.48 min and two additional peaks with t_R of 9.98 and 12.72 min. The first peak was identical with glucose; the two other peaks, however, could not be attributed unambiguously to known glycosides. Under the conditions of hydrolysis used, confirmed by control experiments, only monosaccharides were generated. Measurements with FAB-MS did not result in the detection of a molecular ion peak, whereas the number of sugar protons as deduced from the ^{1}H NMR spectrum identified the original sugar substituent as a disaccharide.

Compounds **7** and **8** were identified as 5,6,3′,4′-tetrahydroxy-7-methoxyflavonol 3-*O*-disaccharides (Figure 2). The EI/MS spectrum of compound **7** (and **8**) gave a base peak at *m*/*z* 332 (A^+) and major fragment ions with *m*/*z* 314, 303, and 289. The latter signal with $M^+ - 43$ is typical for a ketone possessing an aryl-OCH$_3$ function. The ^{1}H NMR spectrum of compound **7** showed signals at δ 6.44 (1H, s, 8-H), 7.50 (1H, d, ^4J = 2.5 Hz, 2′-H), 6.78 (1H, d, ^3J = 9.5 Hz, 5′-H), 7.52 (1H, q, ^3J = 9.5 Hz, ^4J = 2.5 Hz, 6′-H), 12.70 (1H, s, 5-OH), 9.20 (1H, s, 3′-OH), 9.80 (1H, s, 4′-OH), 10.75 (1H, s, 6-OH), 3.75 (3H, s, 7-OCH$_3$), 5.35 (1H, d, ^3J = 7.5 Hz, 1″), 4.00 (1H, d, ^3J = 9.5 Hz, 2″), 2.80−3.70 (other sugar protons). The ^{1}H NMR spectrum of compound **8** was identical with that of compound **7** except for the signals at 7.62 (1H, q, 6′-H) and 7.53 (1H, d, 2′-H). This difference can be caused only by the glycosidic substituent at C-3. This substituent could not be identified unambiguously, for the same reasons as outlined for compound **6**. Again, there were no differences with respect to the FT-IR and UV−vis spectra. Compounds **7** and **8** showed absorption bands with λ_{max} of 207, 259, 269, and 346 nm.

Fraction 1 from preparative HPLC (Figure 1) comprised two additional compounds with antimutagenic activities, designated compounds **9** and **10,** according to micropreparative HPLC, procedure I (t_R of 6.36 and 6.89 min). Analytical HPLC, procedure VI, showed a t_R of 11.18 min for compound **9** and a t_R of 11.49 min for compound **10**. The ^{1}H NMR spectrum and UV−vis spectrum of compound **9** were identical to those of 5,8,4′-trihydroxyflavanone (*16*) (Figure 2), whereas the respective spectral data of compound **10** were equivalent to those of 7,8,4′-trihydroxyflavanone (*17*) (Figure 2).

When fraction 3 of Figure 1, obtained by preparative HPLC, was subjected to micropreparative HPLC, according to procedure I, 4 major and at least 11 minor peaks were observed, but only 1 peak, t_R of 10.09 min, contained a compound, designated compound **11**, with antimutagenic activity. Again, the same procedure, this time HPLC performed according to procedure II, resulted in the detection of two major peaks, t_R of 6.74 min (compound **13**) and t_R of 7.36 min (compound **12**), with antimutagenic activities and at least 15 additional but inactive minor peaks. Analytical HPLC, procedure VI, indicated the following t_R values: compound **11**, 12.19 min; compound **12**, 13.27 min; and compound **13**, 12.75 min.

Compounds **11**−**13** were available in purities of >90% for instrumental analysis. The ^{1}H NMR spectrum of compound **11** in DMSO-d_6 gave the following signals: 7.56 (1H, s), 7.50 (1H, d, ^3J = 9.5 Hz), 7.45 (1H, d, ^3J = 18 Hz), 7.35 (1H, d, ^3J = 9.5 Hz), 7.10 (1H, d, ^3J = 9.5 Hz), 6.85 (1H, d, ^3J = 9.5 Hz), 6.80 (1H, d, ^3J = 9.5 Hz), 6.48 (1H, s), 6.25 (1H, d, ^3J = 18 Hz), 12.80 (1H, s, OH), 10.70 (1H, s, OH), 9.60 (1H, s, OH), 9.20 (1H, s, OH), 9.70 (1H, s, OH), 3.75 (3H, s, OCH$_3$), 3.90 (3H, s, OCH$_3$). In addition, signals between 3.8 and 2.10 were detected, which were attributed to sugar protons. Compound **11** showed absorption bands with λ_{max} of 212, 251, 272, 304 (shoulder), and 334 nm. The ^{1}H NMR spectrum of compound **12** showed the following signals: 7.90 (1H, s), 7.50 (1H, d, ^3J = 9.5 Hz), 7.50 (1H, d, ^3J = 18 Hz), 7.35 (1H, d, ^3J = 9.5 Hz), 7.10 (1H, d, ^3J = 9.5 Hz), 6.85 (1H, d, ^3J = 9.5 Hz), 6.85 (1H, d, ^3J = 9.5 Hz), 6.58 (1H, s), 6.20 (1H, d, ^3J = 18 Hz), 12.80 (1H, s, OH), 10.70 (1H, s, OH), 9.85 (1H, s, OH), 9.65 (1H, s, OH), 3.90 (3H, s, OCH$_3$), 3.85 (3H, s, OCH$_3$), 3.70 (3H, s, OCH$_3$). In addition, signals between 3.8 and 2.10 were detected that were attributed to sugar protons. Compound **12** showed absorption bands with λ_{max} of 212, 249, 273, 298 (shoulder), and 333 nm. The ^{1}H NMR spectrum of compound **13** differed from that of compound **11** only with respect to a signal at 9.7 (s, OH), which was absent in the spectrum of this compound. Compound **13** showed absorption bands with λ_{max} of 209, 258, 275, 316, and 360 (shoulder) nm. The UV−vis spectra of compounds **11**−**13** indicated a flavonoid structural type. Assignment to a known parent compound (aglycon) was, however, not possible due to the complexity of the chemical shifts and couplings as well as the high number of aromatic H (9 H), OH (4−5), and OCH$_3$ (2−3) groups present. Compounds **11** and **12**, however, differ only in the way that one hydroxyl group of compound **11** has been methylated in compound **12**, whereas compound **13** has one hydroxyl function less than compound **11**. All three compounds contain a sugar moiety the structure of which could not be elucidated due to the small amounts of isolated materials. Nevertheless, according to ^{1}H NMR spectra a glucose moiety is present and the coupling constant J < 7.4 Hz of the proton at 1″ may indicate a β-glycosidic bonding to the aglycon.

DISCUSSION

Spinach is devoid of flavonoids widespread in most plants, such as kaempferol, quercetin, myricetin, apigenin, and luteolin, but contains unique compounds. These are patuletin (quercetagetin 6-methyl ether) and spinacetin (quercetagetin 6,3′-dimethyl ether) (*18*), a glycoside with an aglycon tentatively identified as 3-methoxy-6,7-methylenedioxyquercetagetin (*19*), spinatoside, 3,6-dimethylquercetagetin 4′-*O*-glucuronide (*20*), the 4′-glucuronides of 5,7,4′-trihydroxy-3,6,3′-trimethoxyflavone, 5,3′,4′-trihydroxy-3-methoxy-6,7-methylenedioxyflavone, and 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone (*21*), and patuletin 3-gentiobioside, patuletin 3-glucosyl-(1→6)-[apiosyl-(1→2)]-glucoside, and spinacetin 3-gentiobioside (*22*). In our own investigations we have identified three so far unknown

Table 1. Antimutagenic Potencies of Compounds Isolated from Spinach (*S. oleracea*) on Mutagenicity Induced by 2-Amino-3-methylimidazo[4,5-*f*]quinoline in *S. typhimurium* TA 98

compd	yield (mg/100 g)	IC_{50}[a] (nmol/mL of top agar)	maximum[b] reduction of mutagenicity	aglycon[c] of compd	IC_{50}[a] (nmol/mL of top agar)	maximum[b] reduction of mutagenicity
1	300	49.9	79.0	**1**	54.0	73.7
2	59	38.7	80.0	**2**	40.1	77.3
3	21	58.2	64.7	**3**	53.7	70.1
4	69	44.7	79.2	**4**	47.7	74.2
5	21	24.2	79.1	**5**	24.8	77.1
6	17		38.7	**6**	10.4	79.6
7	56		34.3	**7**	13.0	65.0
8	70		39.5	**8**	13.0	70.1
9	45	not reached	21.6[d]			
10	45	not reached	31.2[d]			
11	33		40.0	**11**		75.4
12	21		49.1	**12**		69.5
13	10.5		21.8	**13**		59.1

[a] IC_{50} is the concentration of a flavonoid in nmol/mL of top agar (μM) required to inhibit the mutagenic activity by 50%, calculated from corresponding dose−response curves. [b] Maximum concentration tested was 77.9 μg/mL of top agar unless otherwise indicated. [c] After chemical hydrolysis. [d] Maximum concentration was 93.5 μg/mL.

6,7-methylenedioxyflavonol glucuronides (compounds **1**−**3**) with hydroxyl and/or methoxyl groups at C-5, C-2′, C-3′, and C-4′. These compounds fit into the group of methylenedioxyflavonoids with substitution at C-6/C-7 and/or at C-3′/C-4′ (*23*). Two other flavonol glucuronides (compounds **4** and **5**) described here for the first time were methyl ether derivatives of quercetagetin (3,5,6,7,3′,4′-hexahydroxyflavone) and may be formally generated by opening of the five-membered dioxolane ring system at C-6/C-7 from compounds **1** and **3**. In spinach samples investigated by us, the glucuronide moiety was always linked to the hydroxyl group at C-3, in contrast to other researchers who had described various 4′-glucuronides (*24*). Identification of flavonol glycosides by us agreed in part with the results of Aritomi et al. (*21*). With respect to sugars we detected only disaccharides, comprising glucose and two unidentified glycosides, whereas ref *21* identified the disaccharide gentiobiose and a new trisaccharide. Gentiobiose with two glucose molecules 1→6 linked might well be one of our incompletely characterized disaccharides. In general, the basic oxygenated structure pattern of quercetagetin was detected by all groups and the 6,7-methylenedioxy derivative by most investigators as well as the existence of glucuronides and 3-hydroxy linked saccharides. There is, however, major disagreement with respect to methylation of hydroxyls of quercetagetin and 6,7-methylenedioxyquercetagetin between us and others. In addition, various flavonoids of so far unknown structure seem to exist in spinach (*19*, *22*, this investigation). In our own investigations, we detected glycosides with aglycons which resemble typical flavonoids but must comprise more complicated systems with a total of six to seven hydroxyl and methoxy groups. Finally, two flavonoids present as aglycons were identified as well-known flavanones: 5,8,4′-trihydroxyflavanone (compound **9**) (*16*) and 7,8,4′-trihydroxyflavanone (compound **10**) (*17*).

The aim of this research was to isolate and characterize from spinach compounds with antimutagenic activities against 2-amino-3-methylimidazo[4,5-*f*]quinoline. Antimutagenic potencies of flavonoids detected here were compared with those of a series of 64 flavonoids investigated previously (*13*). It was confirmed that glycosides, among them flavonol 3-glycosides, were less potent than the corresponding aglycons (compounds **6**−**8** and **11**−**13**). Because various flavonoid glycosides in contrast to the respective aglycons did not exert any antimutagenicity, additional glycosides may exist in spinach but have remained undetected in these investigations. The subgroup of flavonol 3-glucuronides, however, did not differ from their aglycons in antimutagenic potency (compounds **1**−**5**, Table 1). The 6,7-methylenedioxy function in quercetagetin derivatives seems to have no influence on antimutagenicity (compounds **1**−**3** versus **4** and **5**). The introduction of a hydroxyl/methoxy function at C-6, however, considerably reduced antimutagenic potency: IC_{50} values of flavonol, 6-methoxyflavonol, and quercetin (3,5,7,3′,4′-OH) were 0.67, 4.0, and 3.2 μM, respectively, whereas quercetagetin with an additional 6-OH function was inactive. Therefore, patuletin will probably lack antimutagenicity against this imidazoquinoline as well as spinacetin glycosides and may not be detectable in our test system. However, a total of four mono-, di-, and trimethyl ethers of quercetagetin exerted antimutagenicity, the potencies of which (IC_{50} = 25−55 μM) were somewhat lower than those of morin, robinetin, and myricetin (3,5,7,3′,4′,5′-OH). In addition, due to the inactivity of quercetagetin, its 6,7-methylenedioxy derivative and their glucuronides, if present, would not be detected. Flavanones with an 8-hydroxyl group were not available as reference compounds for antimutagenicity testing. However, such a hydroxyl group may reduce antimutagenic potency considerably because flavanones with a 5,7,4′-hydroxyl substitution pattern (naringenin) and related compounds exerted strong antimutagenicity against heterocyclic amines.

By means of activity-linked enrichment of antimutagenic compounds from nutritional and medicinal plants, applying the same procedures as in these investigations, various flavonoids have been isolated and identified. The flavonoids luteolin, galangin, and quercetin were isolated from peppermint (*Mentha piperita*), sage (*Salvia officinalis*), thyme (*Thymus vulgaris*) (*25*), and oregano (*Origanum vulgare*) (*26*), respectively, and, in addition, cirsimaritin and salvigenin from rosemary (*Rosmarinus officinalis*) (*27*) as well as genkwanin, cirsimaritin, hispidulin, and apigenin from carqueja (*Baccharis trimera*) (*28*), a Brazilian folk medicine plant, and were shown to exert antimutagenicity against 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole. 3-Kaempferyl *p*-coumarate from bay laurel (*Laurus nobilis*), however, was a novel compound (*29*). In previous investigations of plant extracts from fruits and vegetables, we showed that spinach juice, as well as

various solvent extracts from residues, exerted especially strong antimutagenic effects against 2-amino-3-methylimidazo[4,5-*f*]quinoline (*11*, *12*). [Strong protective effects of spinach were also exerted against in vivo clastogenicity of benzo[*a*]pyrene and cyclophosphamide in mice (*8*).] In the residues, a series of active compounds of unknown structure were present, among them basic and acidic substances, but at that time chlorophyll was the only antimutagen identified. Within the limits of experimental error, ~50−100% of the total antimutagenicity of original spinach juice ($IC_{50} = 8.4 \pm 4.1$ μL) (*11*) can be explained by the presence of the flavonol glucuronides, compounds **1**−**6** (525 mg/100 g of spinach; $IC_{50} = 50$ μM; 4.6 g of solids/100 mL of spinach juice) (*30*).

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