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Isolation and Identification of the First C-17 Limonin Epimer, Epilimonin

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Limonoids are a family of highly oxygenated triterpenoid secondary metabolites found in significant quantities in *Citrus* and reported to possess multiple health promoting properties. This is the first known report of the isolation and characterization of an epimer of limonin. The epimer, named epilimonin, was isolated by fractional crystallization from a mixture consisting mainly of limonin and epilimonin obtained as byproduct from our efforts to isolate limonin glucoside. Side-by-side comparison of the MS, IR, and ^{1}H and ^{13}C NMR data of epilimonin and limonin lead to the assignment of C-17 as the site of epimerization. An earlier study on the bioavailability of limonin glucoside in humans had indicated that limonin glucoside was metabolized to give limonin and a second limonin metabolite. Results from analyzing epilimonin by the same chromatographic conditions used for the bioavailability study suggest that the second limonin metabolite was epilimonin.

KEYWORDS: ***Citrus*; limonoids; epimer; limonin**

INTRODUCTION

Limonoids are a family of highly oxygenated triterpenoid secondary metabolites found in significant quantities in *Citrus* and reported to possess multiple health promoting properties (*1*–*4*). These secondary metabolites are found in a variety of citrus tissues as aglycones, glucosides, or A-ring lactones, the metabolic precursors to limonoid aglycones and glucosides. Limonin and limonin glucoside are the most abundant aglycone and glucoside for most citrus species (*5*). Recently, work in our laboratory has focused on the isolation of multigram quantities of limonin glucoside. As a byproduct of our efforts, we also isolated an aglycone rich fraction that consisted mainly of a mixture of limonin (CAS registry number 1180-71-8) and an unknown compound with the same molecular mass as limonin (HPLC-ESI/MS *m*/*z* 471.1 $[M + H]^{+}$).

In 2003, we reported a study in which 16 participants received a single dose of limonin glucoside (0.25−2.00 g) in order to evaluate the human bioavailability of limonin glucoside in humans (*6*). Results from this study indicated that limonin glucoside was metabolized to give limonin and a second metabolite with similar chromatographic behavior and mass spectrum as limonin. The unknown was proposed to be an epimer of limonin formed through the inversion of C-17 (**Figure 1**). However, a lack of sufficient materials precluded the isolation and formal characterization of the metabolite. The goal of this study was first to isolate and characterize the unknown compound found in the third fraction obtained from the purification method in order to determine its structure and second to determine if this compound was the same unknown compound detected during the bioavailability study.

MATERIALS AND METHODS

Materials and Chemicals. Solvents (acetonitrile, chloroform, methanol) were HPLC grade and along with formic acid (88%, ACS reagent grade) were purchased from Fisher Scientific Ltd. (Waltham, MA). Ethyl alcohol (190 proof, USP grade) was purchased from

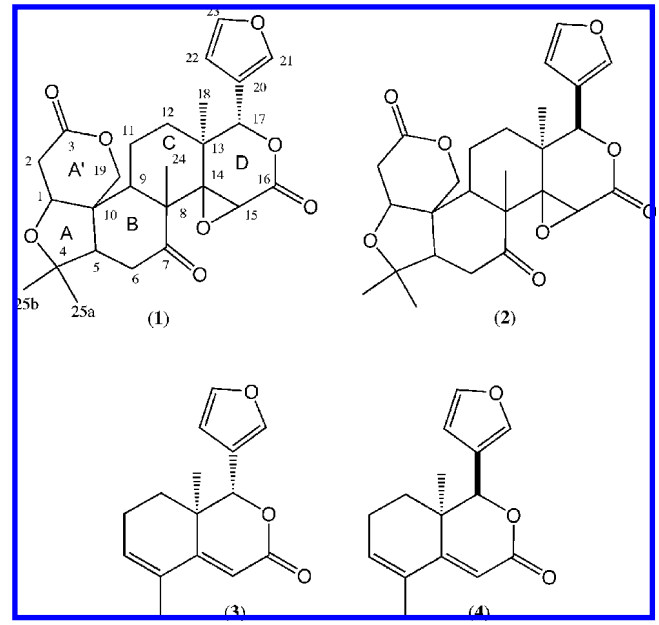

Figure 1. Structures of limonin (**1**), epilimonin (**2**), *dl*-pyroangolensolide (**3**), and *dl*-*epi*-pyroangolensolide (**4**).

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Equistar Chemicals, LP (Tuscola, IL) and filtered through a 0.45 μm type HA membrane filter (Millipore, Billerica, MA) prior to use. Deuterated chloroform (98.8+ % D) was obtained from Sigma-Aldrich (St. Louis, MO). Water was deionized to ≥18.1 MΩ/cm resistance using a Barnstead NANOpure deionization system (Dubuque, IA) and filtered through a 0.45 μm type HA membrane filter (Millipore, Billerica, MA) prior to use. Pure crystalline limonin was available as analytical standard in our laboratory.

Mixtures of limonin and the unknown (epilimonin) were obtained as byproducts from our efforts to isolate multigram quantities of limonin glucoside from citrus molasses. The development and application of this pilot-scale method for the isolation of limonin glucoside will be detailed in a separate manuscript. However, in brief, limonin glucoside was first obtained from citrus molasses using the method of Schoch et al. (*7*). The resulting material was further purified by recrystallization from water. A final polishing of the crystallized material by C-18 flash chromatography yielded an aglycone rich fraction that contained limonin and the unknown (epilimonin).

Instrumentation. A Mel-Temp apparatus from Laboratory Devices, Inc. (Holliston, MA) was used for melting point determinations. For optical rotation determinations, a PerkinElmer, Inc. (Waltham, MA) model 241 polarimeter was used. IR spectra were recorded on a System 2000 FT-IR (PerkinElmer, Inc.). Samples were analyzed by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) using a quadrupole/time-of-flight mass spectrometer (Q-STAR Pulsar I, MDS Sciex/Applied Biosystems, Toronto, Canada).

NMR data were observed at 300 K from samples in $CDCl_3$ with TMS as an internal standard on a Bruker model ARX400 spectrometer, equipped with a 5 mm broadband, multinuclei, triple-axis gradient probe, at a frequency of 100.62 MHz for carbon and 400.13 MHz for proton. A 30° pulse at a 2.3 s repetition rate was used for carbon, and a 90° pulse at a 7−8 s repetition rate was used for protons. Spectral data processing was carried out by the Bruker Xwinnmr-2.6 software package.

For spectra calculation, a line-broadening factor of 0.1 and 1.0 Hz were assigned to proton and carbon, correspondingly. The spectra were processed with a manual phase correction, followed by an automatic baseline correction, and setting TMS manually as the spectral origin. A manual peak integration procedure was applied. The attached protons for ^{13}C signals were established by DEPT90 and DEPT135 assays. One- and two-dimensional experiments were run for both nuclei. Spectral assignments were based on the homo- and heterocorrelations observed in COSY, HETCOR, and NOESYTP experiments and comparison with spectra of related limonoids.

Isolation of Epilimonin through Fractional Crystallization. A mixture of limonin and epilimonin, dissolved in 50% EtOH, was slowly reduced in volume from 700 to 350 mL over 3 d. The resulting precipitate was stirred using a spatula, and the suspension was filtered through a Whatman #1 filter paper using a Büchner funnel and house vacuum. Upon standing for 2 d at ambient temperature (21 °C), additional precipitate formed. The mother liquor was decanted, and the precipitate was redissolved in EtOH (35 mL) and left standing covered with punctured aluminum foil. The solvent was allowed to slowly evaporate, yielding crystals. Reaching near dryness after approximately three months, the residual supernatant (0.8 mL) was removed using a pipet. The crystals were then suspended in 10 mL of EtOH to wash them. After 3 d of equilibration, the supernatant was removed, and the crystals were air-dried to give colorless truncated cubes (15.4 mg). Attempts to obtain crystals of sufficient size for X-ray crystallographic analysis were unsuccessful.

HPLC Analysis of Limonin and Epilimonin. HPLC was used to monitor the progress of the crystallization process and was accomplished using a binary gradient composed of (A) acetonitrile and (B) formic acid (25 mM) that resolved epilimonin and limonin. Analyses were conducted on a Waters 2695 system coupled to a Waters model 996 photodiode array detector (190−250 nm) and Sedex 55 ELS detector (50 °C, 2.5 bar N_2, S.E.D.E.R.E., Alfortville, France). Instrument control and data acquisition were accomplished using MassLynx (version 4.0). For some experiments, a PDR-Chiral, Inc. advance laser polarimeter (Lake Park, FL) was also used. Signals from the ELS detector or in-line polarimeter were acquired in MassLynx through a Waters SAT/IN Module. A 50 mm × 2 mm Phenomenex Phenosphere-Next-5 μ phenyl column (Torrance, CA) equipped with a guard column of the same stationary phase was used and maintained at 30 °C. The flow rate was 0.5 mL/min, and the column was eluted starting with a linear gradient from 15/85 (A/B) to 30/70 (A/B) in 5 min, continuing with an isocratic stretch at 30/70 (A/B) for 5 min, followed by a linear gradient in 2 min back to 15/85 (A/B) and re-equilibrating at this composition for 3 min. The total run time was 15 min. Sample injection volumes were 20 μL.

HPLC-ESI/MS Analysis. HPLC-ESI/MS analysis was conducted on a system controlled by Xcalibur (version 1.4) that consisted of a Waters 2695 coupled to a TSP UV 2000 detector (210 nm) and Thermo Finnigan LCQ Advantage ion-trap mass spectrometer (San Jose, CA). The same binary gradient described above was utilized with 10 μL injections. The flow from the TSP UV 2000 detector was directed through a LC Packing's Acurate (Sunnyvale, CA) flow splitter, such that only $^1/_5$ of the flow was introduced into the mass spectrometer. MS analysis was conducted in positive mode. The mass spectrometer was tuned through optimization on the signal generated by introduction of limonin (5 ppm, positive mode, *m/z* 471.1) solution into the mass spectrometer in the LC mobile phase at the flow rate used for analysis. Following tuning, the mass spectrometer was operated with a capillary temperature of 380 °C, spray voltage of 4.50 kV, capillary voltage of 3.0 V, and scan range 375−975 *m/z*. For MS/MS experiments, the spectrometer was set to trap molecular ions of 471.1 ± 0.5 *m/z* and to use a fragmentation energy of 36%.

HPLC Analysis Confirming Peak Identity from Bioavailability Study. The same HPLC systems as described above were used; however, the conditions (solvents, stationary phase, flow rate, etc.) were the same as those used for the bioavailability study (*6*). In brief, a Hypersil BDS C-18 (3 μm, 2 mm × 50 mm) reversed phase column (Keystone, Bellefonte, PA) and guard thermostatted at 40 °C and flowing at 0.3 mL/min were used. The injection volume was 20 μL, and the solvents were (A) 4 mM FA in MeOH and (B) 4 mM FA in H_2O. The elution started with 30/70 (A/B). At 2 min, the ratio was stepped up to 40/60 (A/B), and this ratio was held for 3 min, followed by a linear gradient to 70/30 (A/B) within 5 min. This composition was held for 2.3 min followed by re-equilibrating at 30/70 (A/B) for the remaining 4.7 min. The total run time was 15 min.

Epilimonin. Colorless crystals (EtOH), mp 316−318 °C with decomposition; $[\alpha]^{23}_D$ +32° (*c* 0.134, Me_2CO); IR (KBr) υ_{max} 2955, 1760, 1732, 1708 cm^{-1}; ^1H and ^{13}C NMR, see **Table 1**; ESI-MS *m/z* 471.1 $[M + H]^+$; HR-ESI-MS *m/z* 493.1810 $[M + Na]^+$, calcd for $C_{26}H_{30}O_8Na$, 493.1838.

Limonin. Colorless crystals (i-PrOH-CH_2Cl_2), mp 284−295 °C with decomposition; $[\alpha]^{23}_D$ −124.7° (*c* 0.121, Me_2CO); IR (KBr) υ_{max} 2968, 1760, 1709 cm^{-1}; ^1H and ^{13}C NMR, see **Table 1**; ESI-MS *m/z* 471.1 $[M + H]^+$; HR-ESI-MS *m/z* 493.1817 $[M + Na]^+$, calcd for $C_{26}H_{30}O_8Na$, 493.1838.

RESULTS AND DISCUSSION

As the starting point for our efforts to isolate and characterize the unknown compound, we began with an aglycone rich fraction obtained from our polishing of limonin glucoside by C-18 flash chromatography for the removal of limonin. Analysis by high-performance liquid chromatography coupled to photodiode array and evaporative light scattering detectors (HPLC-PDA-ELSD) showed that the fraction contained limonin and the unknown in equal proportions. The same HPLC method was also utilized to monitor our efforts to isolate the unknown.

Slow reduction of the solvent volume of this fraction to half its original volume resulted in the formation of large colorless shiny platelike precipitates. HPLC analysis of the precipitate and the residual solvent indicated that the precipitate was limonin and that the unknown still remained in solution. A few hours after removing these solids, a precipitate made up of much smaller particles was observed at the bottom of the flask, and after standing for 2 d, a crème-colored precipitate consisting

Table 1. ^{1}H and ^{13}C NMR Data for Compounds **1** and **2** in $CDCl_3$

	^{1}H		^{13}C	
position	limonin (**1**)	epilimonin (**2**)	limonin (**1**)	epilimonin (**2**)
1	4.03 (1H, m)	4.11 (1H, m)	79.2	79.2
2a	2.67 (1H, dd, 16.8, 2.0)	2.62−2.76 (1H, overlapping)	35.7	35.6
2b	2.98 (1H, dd, 16.8, 4.0)	2.98 (16.8, 4.0)		
3			169.0	169.0
4			80.3	80.5
5	2.22 (1H, dd, 15.8, 3.4)	2.29 (1H, dd, 16.0, 3.2)	60.7	58.9
6a	2.46 (1H, dd, 14.4, 3.2)	2.44 (1H, dd, 16.6, 3.6)	36.4	36.3
6b	2.85 (1H, dd, 15.8, 14.6)	2.62−2.76 (1H, overlapping)		
7			206.0	206.0
8			51.4	49.6
9	2.55 (1H, dd, 12.2, 3.0)	2.62−2.76 (1H, overlapping)	48.2	46.6
10			46.0	45.6
11	1.72−1.95 (2H, m)	1.73−1.86 (1H, m)	19.0	18.5
11b		1.89−2.01 (1H, m)		
12	1.46−1.58 (2H, m)	1.45−1.61 (2H, m)	30.9	27.3
13			38.0	40.3
14			65.7	69.0
15	4.05 (1H, s)	4.63 (1H, s)	53.9	55.5
16			166.5	167.8
17	5.47 (1H, s)	4.88 (1H, s)	77.8	85.0
18	1.18 (3H, s)	1.56 (3H, s)	20.7	28.2
19a	4.76 (1H, d, 13.0)	4.67 (1H, d, 13.2)	65.4	64.9
19b	4.46 (1H, d, 13.0)	4.49 (1H, d, 13.2)		
20			120.1	122.1
21	7.40 (1H, m)	7.49 (1H, m)	143.3	143.4
22	6.34 (1H, m)	6.56 (1H, m)	109.7	111.9
23	7.41 (1H, m)	7.37 (1H, m)	141.2	142.8
24	1.08 (3H, s)	1.14 (3H, s)	17.7	19.5
25a	1.29 (3H, s)	1.31 (3H, s)	30.2	30.4
25b	1.18 (3H, s)	1.17 (3H, s)	21.4	21.6

largely of the unknown and only little limonin was collected. This precipitate was taken up in EtOH and left standing covered with punctured aluminum foil. After 3 months, the solvent level had been reduced to 0.8 mL, and precipitates of different sizes, small cubelike crystals and powderlike materials, had formed. Fresh EtOH was added to dissolve the smaller precipitates, and the remaining cubelike crystals were manually collected to yield the unknown (15.4 mg) free of limonin.

Following isolation of the unknown, a side-by-side comparison of the physical properties, including melting point, MS, optical rotation, IR, and ^{1}H and ^{13}C NMR, of the unknown and limonin was completed. The unknown melted with reddish-brown decomposition at 316−318 °C, 18−20 °C higher than the reported melting point (298 °C) for limonin, and 21 °C higher than the limonin sample used in the present study. LC-MS analysis of the unknown and limonin had shown that they both exhibited the same molecular ion of 471.1 *m/z*. The exact masses ($[M + Na]^{+}$) obtained by HR-ESI-MS correlated well with the theoretical values and confirmed that the unknown and limonin were of the same elemental composition. MS/MS experiments yielded nearly identical fragmentation patterns, suggesting that the connectivity within the structures was the same. In addition, the dominant daughter ions observed (425.2 and 367.1 *m/z*) were the same as those observed in the bioavailability study (*6*).

We then turned to optical rotation and IR analyses to probe the possibility that the difference between the two compounds resulted from a change in the orientation of one or more stereocenters. Initial investigation of the optical rotation of the unknown using an in-line polarimeter and its subsequent determination using a standard polarimeter indicated that the unknown possessed a (+) rotation, whereas limonin exhibited a (−) rotation. The IR spectrum also showed a striking difference. While authentic limonin showed two maxima at υ_{max} 1760 and 1709 cm^{-1} in the carbonyl region, consistent with the reported values, the unknown showed three distinct maxima in this region (υ_{max} 1760, 1732, and 1708 cm^{-1}). This difference may be explained as follows. First, absorbance corresponding to 1732 may be present in limonin also, but only as a weak shoulder, and partially obscured by the higher intensity and width of the 1760 band (for spectrum see Supporting Information). Second, a downward shift may have resolved the central band. Such a downward shift can be identified for a single carbonyl band in a previous report (*8*) on an analogous epimeric pair: *dl*-pyroangolensolide (**3**) and its epimer (**4**), shifting from 1710 to 1690 cm^{-1}. At this stage, we felt we had sufficient data to conclude that the unknown was an epimer of limonin and that the altered stereocenter(s) was likely located in close proximity of one of the three carbonyls found in the structure.

^{1}H and ^{13}C NMR assignments were made on the basis of 1- and 2-D experimental data acquired for limonin and the proposed epimer and spectral data existing for other limonoids. The proton and carbon NMR data (**Table 1**) reaffirmed that the compounds were of the same basic skeleton and suggested that the D-ring was the probable site of epimerization, since the most apparent differences in the spectra were associated with the chemical shifts of H-15, H-17, and H-18, and carbons C-17 and C-18. In comparison to limonin, the H-18 methyl singlet of the epimer shifted downfield 0.38 ppm. A downfield shift of similar intensity (0.34 ppm) was observed for the analogous methyl group when *dl*-pyroangolensolide (**3**) was compared to *dl-epi*-pyroangolensolide (**4**) (*8*) (**Figure 1**), providing evidence that C-17 is the site of inversion in epilimonin. Inversion at this site would also result in the placement of an H-17 methine proton in a deshielded position, since it would no longer be in close proximity of the epoxide ring and is in line with the 0.59 ppm upfield shift observed for H-17. NOE experiments provided further confirmation to the inversion of C-17 (**Figures 2** and **3**). For limonin, a strong NOE interaction between the H-18 methyl and the furan protons was observed, whereas for epilimonin, this same interaction was absent and interactions between H-24 and H-22 and between H-18 and H-17 were visible. On the basis of these conclusions, we named the unknown compound epilimonin.

Because results from the human bioavailability study had demonstrated that limonin glucoside was metabolized to give at least limonin and a second metabolite with similar chromatographic behavior and mass spectrum as limonin (*6*), we compared the elution behavior of epilimonin under the same chromatographic conditions used for that study to determine if epilimonin and the observed unknown were one and the same. The unknown metabolite was reported to have eluted approximately 0.9 min before limonin. Under the same chromatographic conditions epilimonin (RT: 8.59 min) was found to elute 0.8 min before limonin (RT: 9.36 min). With these positive results, we had hoped to make a direct comparison to samples from the bioavailability study but regardless of our best efforts found that the retained samples had deteriorated beyond use. However, we believe that the similarity in elution order and relative retention times combined with the identical MS/MS data for the two compounds provides a convincing argument that the unknown observed in human plasma was epilimonin.

In summary, we have isolated and characterized the first known epimer of limonin, epilimonin. A combination of

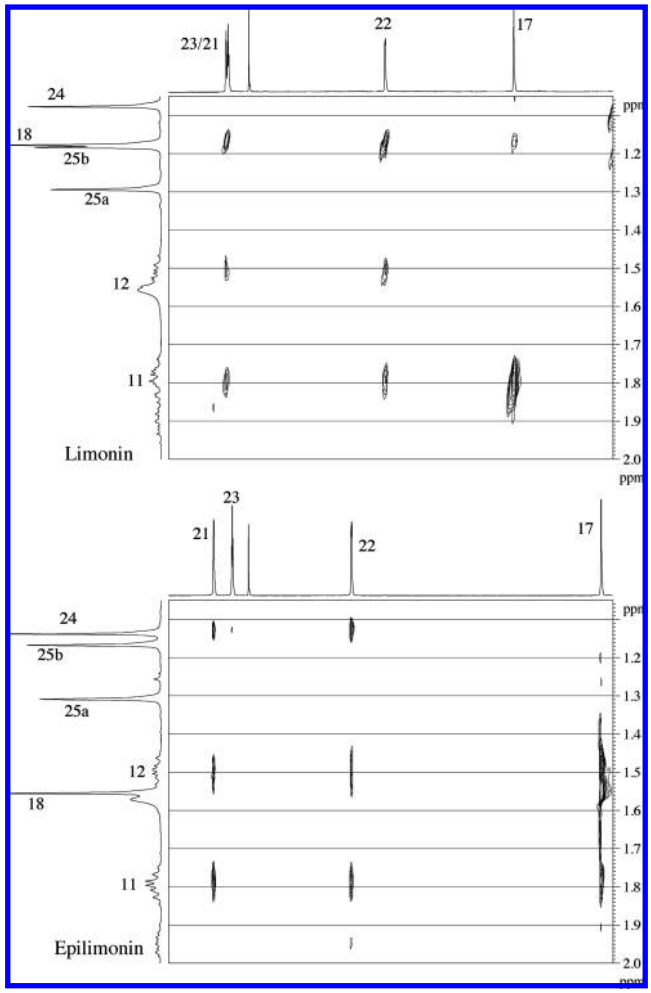

Figure 2. Key NOE enhancements for limonin (**1**) and epilimonin (**2**).

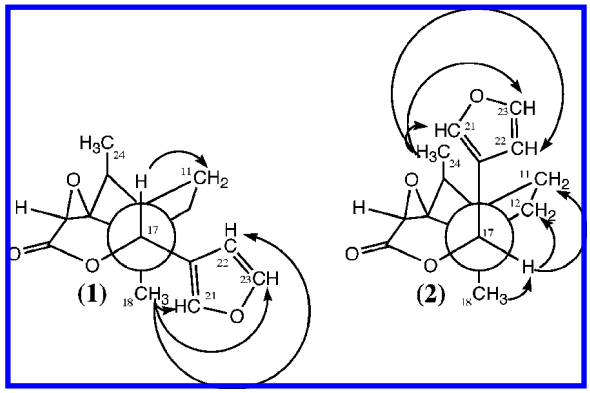

Figure 3. Newman projections and numbering of partial structures of limonin (**1**) and epilimonin (**2**) showing key NOE enhancements.

analytical methods was used to identify C-17 as the site of inversion and demonstrate that epilimonin was likely the same unknown metabolite observed in a recently reported human bioavailability study. Considering that epilimonin was the dominant metabolite for the first 1−3 h following ingestion of limonin glucoside, we are continuing our isolation efforts in order to obtain sufficient materials to conduct structural activity studies to evaluate how the inversion at C-17 affects its biological activities. Other areas that warrant investigation included epilimonin's taste profile and whether the compound is endogenous to citrus or just byproduct of human metabolism or the method used to isolate limonin glucoside.

ABBREVIATIONS USED

NMR, nuclear magnetic resonance; HPLC-MS, high-performance liquid chromatography coupled to mass spectrometry; HPLC-ESI/MS, HPLC coupled to mass spectrometry with electrospray ionization; HPLC-PDA-ELSD, HPLC coupled to photodiode array and evaporative light scattering detectors; RT, retention time.

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Supporting Information Available: IR and NMR data for the compounds limonin and epilimonin. This material is available free of charge via the Internet at http://pubs.acs.org.

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