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Isolation and structural characterization of dihydrobenzofuran congeners of licochalcone A

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Abstract

In an effort to explore the residual complexity of naturally occurring chalcones from the roots of *Glycyrrhiza inflata* (Fabaceae), two new licochalcone A (LicA) derivatives were isolated as trace metabolites from enriched fractions. Both constituents contain a dihydrofuran moiety linked to carbons C-4 and C-5 of the retrochalcone core. Compound **1** (LicAF1) represents a new chemical entity, whereas compound **2** (LicAF2) has previously been reported as a Lewis acid catalyzed rearrangement of LicA. Evaluation of chirality revealed that both dihydrofuran derivatives existed as a mixture of *R* and *S* enantiomers. Interestingly, when solutions were exposed to sunlight, both dihydrofuran retrochalcones, initially isolated as *trans* isomers, were found to rapidly isomerize yielding *trans* and *cis* isomers. Analysis of the 1D ¹H NMR spectra of the photolysis products revealed the presence of two sets of proton resonances ascribed to each of the geometric isomers. An up-field shift of all proton resonances arising from the *cis* isomer was observed, suggesting that anisotropic shielding effects were introduced through an overall perturbation of the 3-dimensional structure upon photoisomerization. Similar up-field shifts were observed in the ¹³C spectrum of the *cis* isomer, except for the C=O, C- α , and C-6 carbons, which experienced downfield shifts. Analogous NMR results were observed for LicA. Hence, the results presented herein encompass the isolation and full characterization of LicAF analogs **1** and **2**, and observations of their *trans*-to-*cis* photoisomerization through the systematic analysis of their NMR spectra.

Graphical abstract

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Supplementary Material and Original NMR Data: The semi-preparative HPLC chromatograms associated with the isolation of the new retrochalcones (S2-S3), the assigned ¹H NMR spectra of the most abundant licorice compounds also isolated from these fractions (S4), The ¹H NMR spectra of LicA before (S5) and after photo-isomerization (10-day exposure S6), the HiFSA profiles of LicA and its derivatives LicAF1 and LicAF2 (S7, S11, S14); the annotated ¹³C spectra of LicAFs (S9, S12) can be found as freely available supplementary material. The original NMR data (FIDs) of all isolated licorice compounds in this study, as well as the photoisomerized LicA and LicAFs, are made available at doi:10.7910/DVN/DFAVTE (Harvard Dataverse).

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Keywords

Licochalcone A; dihydrobenzofuran; cis/trans isomerization; retrochalcone; residual complexity; raw NMR data

1. Introduction

The roots and rhizomes from *Glycyrrhiza inflata* Batalin are the third most utilized licorice roots after *G. glabra* (European licorice) and *G. uralensis* (Chinese licorice). *G. inflata* is also naturally occurring and cultivated in China (Xinjiang province) [1], where it is communally considered a yellow skin licorice due to the strong yellow-brown coloration of the dried roots compared to the two other major *Glycyrrhiza* species [2]. Such coloration can be attributed to the abundance of diverse chalcones, as the phytochemical composition of *G. inflata* has been demonstrated to greatly differ from the other widely used *Glycyrrhiza* species, due to the presence and abundance of structurally related “retrochalcones”. These compounds are structurally related to echinatin and licochalcone A [3][4][5][6]. In fact, the term retrochalcone has been coined initially for echinatin as a result of a biosynthetic investigation in *G. echinata* callus cultures [7][8] [9]. In these studies, the precursors of echinatin were assigned to isoliquiritigenin and its oxidized form, licodione. Subsequent C-2' methoxylation of licodione followed by a reduction of the adjacent keto group and dehydration was identified as the mechanism that leads to an inversion (thus the prefix, “retro”) of the original A- and B-rings compared to other naturally occurring chalcones [7] [8][9]. Therefore, from a structural point of view, retrochalcones are characterized by the lack of an oxygen functionality at C-2'/C-6' and the presence of a methoxy group at C-2 (Fig. 1A). Licochalcone A (LicA), C, E, and F are isomers, which share the same backbone structure of echinatin, but differ structurally by the position and by the type of aliphatic side chain attached on C-5 (Fig. 1B). Due to the presence of α - β unsaturation, chalcones, and thus retrochalcones, are known to be photoreactive, capable of undergoing photo-induced *trans-to-cis* isomerization through electron delocalization facilitated by the presence of the conjugated carbonyl function [10, 11]. As such, the chemical structure of LicA and its isomers possesses two chemically reactive double bonds: (1) the α - β unsaturation, which absorbs long wavelength light, thereby promoting *trans-to-cis* isomerization; and (2) the unsaturation present in the aliphatic side chain, which can lead to ring closure with the hydroxyl function at C-4. It is generally assumed that the *trans* (E) chalcone isomer tends to be thermodynamically more stable than the *cis* (Z) chalcone isomer. Therefore, chalcone structures in the literature are commonly represented in *trans* configuration [12].

As licorice has been used traditionally since the early beginnings of medical practice, its phytochemical constituents have been widely explored [13][14][15]. Accordingly, the most abundant and, therefore, most straightforward to isolate phytochemicals contained in the most widely used *Glycyrrhiza* species have already been described. Hence, mining for new phytochemicals with a certain degree of structural novelty (new structures) in these well-studied plant materials requires the examination of the residual complexity of their extracts and fractions [16]. Residual complexity encompasses *all* the metabolites present in a sample, which often are obscured by the baseline of chromatographic or ^1H NMR spectral profiles. These compounds are conceived as being barely detectable and, thus, are often overlooked. The objective of the present study was to explore the structural novelty of *G. inflata* retrochalcones by focusing on trace compounds, which collectively contribute to the overall biological properties of *G. inflata* preparations. This report addresses the isolation and structural description of two minor LicA derivatives, including one new compound. Particular attention will be given to the in situ photoinduced *trans*-to-*cis* isomerization of the studied retrochalcones and the influence of the isomerization on both the ^1H and ^{13}C chemical shifts.

2. Materials and Methods

2.1 Preparation of *Glycyrrhiza inflata* crude extract

Roots from wild *Glycyrrhiza inflata* were provided by Dr. Liang Zhao (Lanzhou Institute of Chemical Physics CAS). The roots were additionally identified through macroscopic/microscopic analyses with comparison to voucher specimens at the Field Museum of Natural History (Chicago, IL), as well as DNA barcoding, as previously described [3]. Powdered roots of *G. inflata* (250 g) were extracted by maceration and percolation at room temperature with a solvent mixture composed of ethanol (200 USP proof), *iso*-propanol, and water (90:5:5, v/v) and a plant powder/volume of solvent ratio of 1/15. After concentration, and freeze drying, the extract yielded 12.4% (w/w) of the initial powdered roots.

2.2 Isolation of Licochalcone A derivatives

2.2.1 Countercurrent Separation (CCS)—Fractionation of the crude extract was performed by countercurrent separation (CCS) with the solvent system composed of Hexanes/Ethyl Acetate/Methanol/Water (5:5:5:5 v/v/v) in isocratic, descending mode (reverse phase mode). The ARMEN spot-prep instrument (SCPC-250-B, Armen Instrument SAS, Gilson, Inc.) was filled with the organic upper phase (UP) at a flow rate of 35 mL/min at 500 rpm. The system was then equilibrated at a flow rate of 25 mL/min leading to a S_f of 83% ($V_S = 207$ mL and $V_m = 93$ mL) with a rotation speed adjusted to 2500 rpm. *G. inflata* crude extract (4.240 g), diluted in 20 mL of UP and 20 mL of lower phase (LP) was injected into the column. Fraction collection was set up for 13 mL/fraction. The extrusion was performed after 2.6 column volumes. A total of 82 tubes were collected. The fractions were pooled according to their TLC profiles, leading to a total of 11 final fractions defined as follows: Fraction 1: vials 4-6, fraction 2: vials 7-9, fraction 3: vials 10-13, fraction 4: vials 14-18, fraction 5: vials 19-28, fraction 6: vials 29-33, fraction 7: vials 34-52, fraction 8: vials 53-61, fraction 9: vials 62-66, fraction 10: vials 67-73, fraction 11: vials 74-82. The CS step, as described above, was repeated 3 times in order to fractionate a total of 12 g of crude *G.*

inflata extract. All TLC were performed on Alugram silica gel plates (SiO₂ F₂₅₄, Macherey-Nagel GmbH & Co.), eluted with CHCl₃/MeOH (90:10, v/v) and revealed with 5% H₂SO₄/vanillin reagent. All the eleven fractions were dried in order to calculate the weight recovery as % weight fractions/weight crude extract.

2.2.2 Semi-preparative HPLC—Compounds **1** and **2** were isolated from the fractions 2 (12.7 % w/w crude extract) and 7 (1.9% w/w crude extract), respectively, by semi-preparative HPLC performed on a Shimadzu instruments at 254 and 360 nm. Two different elution systems were developed on a YMC- Pack ODS AQ (1250 x10 mm ID, S-10 µm, 12 mm AQ12S11-251 OWT) column utilizing Acetonitrile (B) and Water (A). For fraction 2, the column was equilibrated with 20% B and eluted for 5 min. The concentration was changed to 45% B over 20 min and held for 10 min, followed by a change to 60% B over 40 min, then held for 10 min, and back to 20% B after 55 min with a flow rate of 1.5 mL/min. Under these conditions, compound **1** eluted at 34.5 min. For fraction 7, elution was initiated with 60% B over 5 min to 65% B over 40 min, held for 5 min, and back to 60% B after 40 min. Under those conditions, compound **2** eluted at 26.5 min. The fractions were prepared at 28 mg/mL and 50-100 µL of solution was injected at each semi-preparative HPLC run.

2.3 Spectroscopic measurements

2.3.1 NMR Spectroscopy—The NMR samples of compounds **1** and **2** were prepared by precisely (0.01 mg) weighing whenever possible the dried material with a Mettler Toledo XS105 Dual Range analytical balance. Sample were dissolved in 200 µL of deuterated solvent such as DMSO-*d*₆, but also CDCl₃, using a Pressure-Lok gas syringe (VICI Precision Sampling Inc., Baton Rouge, LA USA) for accurate volume delivery. From this solution, ~180 µL were transferred into 3 mm standard NMR tubes from Norell (part no. S-3-HT-7, Norell Inc., Landisville, NJ) using calibrated pipets (cat no: 2-000-200, Drummond Scientific, Broomall, PA USA). In order to improve the sensitivity of detection for the acquisition of DeptQ ¹³C as well as HMBC spectra, both compounds were also re-concentrated in 70 µL of DMSO- *d*₆ and analyzed in Shigemi tubes (Shigemi Inc.). All NMR spectra (¹H, ¹³C, gCOSY, gHSQC, gHMBC) were acquired on a Bruker AVANCE 900 MHz equipped with a 5 mm CPTCI probe. All 1D ¹H NMR spectra were acquired at 298 K under quantitative conditions using a 90° single-pulse experiment (AQ = 3.99 sec, D1 = 60.00 sec, P1 = 10.5 µsec, ds = 4, ns = 32, RG = 256). Off-line data processing was performed using the Mnova NMR software package (v.6.0.2, MestreLab Research S.L., A Coruña, Spain). ¹H and ¹³C chemical shifts (δ) were expressed in ppm with reference to the residual solvent signal (DMSO-*d*₅: ¹H spectrum: 2.500 ppm, and ¹³C spectrum: 39.525 ppm). The following processing scheme was used: a mild Lorentzian-to-Gaussian window function (line broadening = -0.3 Hz, Gaussian factor = 0.01) was applied, followed by zero filling to 256k acquired data points before Fourier transformation. After manual phasing, a fifth order polynomial baseline correction was applied. The ¹H-NMR spectrum of compound **2** was also acquired in CDCl₃ at 900MHz for comparison with previously published NMR data [17].

2.3.2 Evaluation of the photoinduced isomerization—To evaluate the photoinduced isomerization of dihydrobenzofuran derivatives of LicA, the NMR samples of both

compounds as well as LicA (Sigma Aldrich, Lot BCBJ5290B, purity: 92.13% w/w) were exposed to ambient daylight for a minimum of 10 days. A sample of LicA was prepared at a concentration of 5.1 mg/mL in DMSO-*d*₆. Quantitative ¹H NMR measurements were acquired before and after light exposure to evaluate the production of the *cis* isomer. PERCH NMR software (v.2013.1, PERCH Solutions Ltd.) was utilized for the iterative ¹H NMR full spin analysis in order to accurately describe chemical shifts, but most importantly coupling constants, and individual line widths (δ , *J*, and *w*) for each new compound and their respective *cis/trans* isomer, as previously described (spin parameter files (.pms) are available in the supplementary materials as well as deposited in the Harvard Dataverse at [doi:http://dx.doi.org/10.7910/DVN/DFAVTE](https://dx.doi.org/10.7910/DVN/DFAVTE)) [18][19]. The differences in the ¹H and ¹³C chemical shifts (δ mean $\pm$ stdv) were calculated between the *trans* and *cis* isomers of the three evaluated structures (compounds **1-2**, and LicA). The structures were optimized in 3 dimensional space inside the MMS module of the PERCH software using the default parameters set up for geometry and energy optimization. The final structural representation was then uploaded in the Free open source software Avogadro (<http://avogadro.cc>) to export the structures for figures.

2.3.3 High resolution MS—LC-MS-MS analysis was carried out using Waters 2695 solvent delivery system connected to Waters SYNAPT quadrupole/time-of-flight (q/TOF) mass spectrometer operated in the positive ion electrospray mode. Separations were carried out using YMC AQ C₁₈ column (2 × 100 mm, 3μm particle size), eluted with a mobile phase consisting of 0.1% FA (solvent A) and MeCN (solvent B) with a linear gradient from 10-95% B over 30 min. The flow rate was 0.2 mL/min and the column was thermostated at 30 °C. High resolution accurate mass measurements (HR-MS) were performed at the resolving power of 10,000 FWHM using Leu-enkephaline as the lock mass. Product ion spectra were recorded at 20 or 25eV using argon as the collision gas. For identification of compounds, molecular compositions and tandem mass spectra were compared with the standard spectra from public (MassBank) and in-house generated databases as well as with spectra published in the primary literature.

2.4 Circular dichroism and polarimetry

Compounds **1** and **2** were diluted in MeOH HPLC grade at a final concentration of 3.94×10^{-2} M. (1 mL final volume). A rectangular quartz cell (Agilent technologies, Part # 5061-3385, 2 mm) was utilized for the Circular dichroism (CD) measurement, which was performed on a Jasco J-815 CD polarimeter. Data were processed using the SpectraManager Software. Additionally, the samples were analyzed using a Perkin Elmer Polarimeter 241 with a 1 cm long cell at room temperature.

3. Results

Mining for structural novelty among the broad collection of already described licorice phytochemicals requires development of fractionation and isolation processes that enable optimal recovery of most of the compounds initially present in the crude extract, so as to efficiently examine its residual complexity. Additionally, such processes should be as simple as possible to minimize the loss of samples between the different fractionation/isolation

steps. To that end, countercurrent separation (CCS) was chosen as the first fractionation step of *G. inflata* extract due to its liquid/liquid nature enabling an optimal recovery (91 % w/w) of the extract initially injected (Supplementary Material S1). As such, CCS of crude *G. inflata* extract produced eleven fractions of decreasing polarities. The isolation of minor constituents from the generated fractions was then performed by semi-preparative HPLC with UV detection at 254 and 360 nm, thereby facilitating a targeted isolation of less abundant chalcones. Compounds **1** and **2** were isolated from fractions 2 and 7, respectively, by semi-preparative reverse HPLC with two different elution methods (see experimental section, and Supplementary Material S2). The most abundant compounds in the selected fractions 2 and 7 were already known characteristic licorice metabolites. Their identity was confirmed through a dereplication process combining 1D, ¹H NMR (at 600 and/or 900 MHz), MS analyses and a comparison of previously published data [20][21][22][23][24]. Hence, licochalcone B [21], liquiritigenin, echinatin, and licoflavone A [23] were the most abundant compounds in fraction 2. Whereas, licoisoflavone A, glabrone [20] and licoflavone B, also called prenyllicoflavone A [22], were the most abundant compounds in fraction 7 (Supplementary Material S4, and raw NMR data at doi:10.7910/DVN/DFAVTE). The weight recovery calculated for compounds **1** and **2** were 0.06 and 0.03% w/w (weight compound/weight crude extract), respectively, clearly indicating that both compounds were found in trace amounts in the original extract. Interestingly, both compounds, which were the least abundant purified compounds from each fraction, displayed a unique MS and 1D ¹H NMR spectra compared to the already known retrochalcones (Figs. 2 and 3). Consequently, further structural analyses were carried out by high resolution LC-MS followed by NMR at 900 MHz. Both 1D (¹H and ¹³C) and 2D (gCOSY, HMBC, HSQC) data were acquired for structural elucidation of compounds **1** and **2**.

High-Resolution MS analysis of **1** indicated a protonated molecule with an *m/z* 355.1545 (Fig. 2A) and an elemental composition of C₂₁H₂₂O₅ (+ 0.6 ppm). High resolution MS analysis, revealed that compound **2** is an isomer of LicA (Fig. 2B), having the same elemental composition determined as C₂₁H₂₂O₄ (+3.5 ppm). The tandem mass spectra of both compounds exhibited a loss of 42 mass units corresponding to the loss of ketene (CH₂=C=O). This fragmentation channel was the same as that observed in the tandem mass spectrum of LicA and is characteristic for chalcones bearing a hydroxy or a methoxy group in the position 2 of the A ring. [25]. Moreover, the fragment ion with *m/z* 121 suggests the presence of a hydroxyphenyl moiety. Interestingly, the LC-MS chromatograms of both compounds revealed two peaks, corresponding to two isomers with the same positive ion MS/MS spectra (Fig. 2). Such observation suggested that each isolated chalcone rapidly isomerizes in solution yielding a pair of *trans* and *cis* isomers as regards the α-β unsaturation. Hence, considering that this isomerization process is generally photoinduced, further isolation of both compounds was conducted in order to protect the sample from light as much as possible prior to NMR analyses. The objective was to produce NMR spectra of mainly one isomeric form, thereby facilitating the structural elucidation process.

Examination of the 1D, ¹H NMR spectra of compounds **1** and **2** revealed identical proton resonances characterized by an AA' XX' pattern centered at 8.03 and 6.88 ppm (2H each, *J* = 8.41 Hz), two sets of doublets corresponding to the α-β unsaturation of a chalcone at 7.98

and 7.72 ppm (1H each, d, $J = 15.5$ Hz, *trans* configuration), and two aromatic singlets at 7.84 and 6.56 ppm. Both compounds were found to have a methoxy group attached to an aromatic ring as indicated by the presence of a singlet at 3.85 ppm singlet (Figs. 3-5 and Table 1). Hence both compounds shared a certain degree of structural similarities related to the aromatic protons with one hydroxyphenyl and a 2-methoxy- or 2-hydroxyphenyl moiety.

The ^1H NMR spectrum of compound **1** (DMSO- d_6 , 900MHz) revealed the presence of a doublet-of-doublets at 4.33 ppm (1H, *dd*, $J = 4.61/7.55$ Hz), a two pairs of doublet-of-doublets at 3.73 ppm (1H, *dd*, $J = 4.61/-11.83$ Hz) and at 3.69 ppm (1H, *dd*, $J = 7.55/-11.83$ Hz) and two singlets (3H, *s*, 1.39 ppm) and (3H, *s*, 1.16 ppm) corresponding to the presence of two methyl groups. The HSQC spectrum of compound **1** revealed the linkage of the doublet-of-doublets at 4.34 ppm with a carbon at 93.07 ppm, suggesting that this carbon is directly bonded to an adjacent oxygen bearing aromatic carbon (Fig. 4). In the gCOSY spectrum, the proton at 4.34 ppm (H-2'') correlated with the two doublets at 3.69 (H-4''a) and 3.73 ppm (H-4''b) both attached to the same carbon at 59.8 ppm (C-4''), thereby suggesting a primary alcohol group (-CH₂OH, C-2''). The HMBC indicated that H-2'', H-4'', the two methyl groups, and the aromatic singlet at 7.84 ppm correlate with a quaternary carbon at 42.10 ppm. Moreover, the two methyl groups and the aromatic singlet at 6.55 ppm gave HMBC correlations with an aromatic carbon at 130 ppm. Hence, the two methyl groups (H₃-5'', and H₃-6'') are attached to the quaternary carbon at 42.10 ppm (C-3''), which is linked to the aromatic carbon at 130 ppm (C-5) and to the carbon at 93.64 ppm (C-2''), thereby demonstrating that a dihydrofuran ring is attached to one aromatic ring. The two aromatic singlets gave HMBC correlations with two aromatic carbons linked to an oxygen: one at 161.77 ppm and the other at 159.60 ppm which also correlated to the methoxy protons at 3.85 ppm (Fig. 4, green and red arrows). Thus, the carbon at 161.77 (C-4) is linked to the oxygen of the dihydrofuran ring, and the methoxy group is attached to the aromatic carbon (C-2, A ring) in the *ortho* position of the α - β unsaturation. Altogether, the ^1H and ^{13}C chemical shifts (Table 1), and the HMBC correlations (Fig. 4) indicate that compound **1** has a dihydrofuran moiety attached to the A ring with a methoxy group bonded in the *ortho* position of the unsaturation. The AA' pattern centered at 8.03 ppm as well as the 15.5 Hz doublet at 7.73 ppm correlated in the HMBC spectrum with the carbonyl function at 187.40 ppm, thereby indicating that the 7.73 doublet occupies the position β to the carbonyl (H- β). The AA' type protons at 8.03 ppm are assigned to the 2'/6' protons of the B ring (Fig. 4, dashed arrows). Generally, HMBC correlations within the B ring that enabled the determination of C-1' and -4' were relatively weak compared to other correlations in the structure. Compound **1** (Figs. 1, 3, and 4) was identified as 3-[2-(hydroxymethyl)-6-methoxy-3,3-dimethyl-2,3-dihydro-1-benzofuran-5-yl]-1-(4-hydroxyphenyl)prop-2-en-1-one. Consistent with the structural similarities to LicA, compound **1** was named Licochalcone A dihydrofuran **1**, abbreviated as LicAF1.

Compound **2** was recognized as an isomer of LicA (Fig. 2). In addition to identical aromatic protons comparatively to LicAF1, 1D ^1H and 2D NMR spectra of **2** showed the presence of an additional quartet at 4.47 ppm (1H, *q*, $J = 6.52$ Hz) which correlated to the doublet at 1.32 ppm corresponding to a secondary methyl group (3H, *d*, $J = 6.52$ Hz). Two other methyl groups present in the molecule were observed as singlets at 1.10 ppm and 1.33 ppm.

According to the HSQC correlations, the quartet appearing at 4.46 ppm (H-2'') is linked to a carbon at 90.2 ppm (C-2''), suggesting that this carbon is attached to an oxygen-bearing aromatic carbon, similar to that found in LicAF1. The two methyl groups at 1.10 and 1.33 ppm (H₃-5'', and H₃-6''), the aromatic singlet at 7.85 ppm (H-6) as well as the methyl doublet at 1.32 ppm (H₃-4''), correlated with C-2'', and with a quaternary carbon at 42.40 ppm, which was attributed to C-3'', also present in LicAF1 (Table 2). In addition, the HMBC correlations within the B ring were observed to be relatively weak, as was observed for LicAF1. However, the series of observed correlations provided evidence for the existence of a linkage between the aromatic ring and the methyl groups through the formation of a dihydrobenzofuran moiety. All the other HSQC/HMBC correlations describing the structure of compound **2** were identical to the correlations observed for LicAF1. Hence, compound **2** (Figs. 1-5) was identified as (2E)-1-(4-hydroxyphenyl)-3-(6-methoxy-2,3,3-trimethyl-2H-1-benzofuran-5-yl)prop-2-en-1-one, and abbreviated LicAF2 in accordance with the structural similarities with LicA and with LicAF1.

Interestingly, LicAF2 has been previously described as a synthetic product obtained from the catalyzed Lewis acid rearrangement of LicA [17]. In this study from Nielsen et al., the NMR data for LicAF2 were reported in CDCl₃. Therefore, the identity of LicAF2 was also confirmed by comparing the reported data in this article with the data obtained from ¹H NMR acquisition in CDCl₃ at 900 MHz (Supplementary Material S13).

According to the proposed structures, both compounds are characterized by a chiral center at C-2''. Hence, measurement of optical rotation was performed to assess chirality. Methanol solutions prepared with each individual compound did not exhibit any deviation of the polarized light, thereby suggesting that the solutions tested consisted of racemic mixtures (Supplementary Material S16).

In order to confirm the photo-induced *trans* to *cis* isomerization and evaluate its impact on ¹H and ¹³C chemical shifts, a second set of 1D ¹H NMR analyses was performed. Both LicAF1 and LicAF2 together with commercial LicA were kept in their individual NMR tubes (DMSO-*d*₆) unprotected from ambient daylight at room temperature (Figs 5-6, **Panel A**). A second set of proton resonances were gradually formed in the acquired 1D ¹H NMR spectra of the three samples in a time dependent manner. Appearance of the additional set of protons, which are assumed to arise from the formation of the *cis* isomer, exhibited an up-field shift when compared to the corresponding *trans* isomer, as also described by Iwata et al. [10]. The second set of protons in both ¹H NMR spectra also exhibited the same cross peak correlation patterns in the 2D spectra. The protons, corresponding to the α-β unsaturated moiety, appeared at 6.47 and 7.05 ppm (³J_{H,H} = 12.8 Hz), respectively, thus confirming the *cis* geometry of the unsaturated double bond and the identity of the produced isomer. To complete the NMR description, iterative ¹H full spin analysis (HiFSA) was performed in order to extract the NMR parameters for each isomeric form. Such HiFSA fingerprinting enables the complete description of coupling constants, line width, but also the molar ratio of *cis* and *trans* isomer for each described structure in the ¹H NMR spectrum [18][19] (Figs. 5 and 6, Sim for simulated spectrum). Moreover, HMBC and HSQC correlations revealed a general up-field shift of all the correlating carbons except for the C=O, C-δ, and C-6, which experienced a downfield shift in the *cis* isomer as compared to

the *trans* isomer (Figs. 5 and 6B). A study of the ^1H and ^{13}C chemical shift differences (δ) between the *trans* and *cis* isomers of the three retrochalcones also revealed that the photoinduced isomerization affected mostly the chemical shifts corresponding to the α - β unsaturation ($\delta \text{H}\alpha = 1.86 \pm 0.02$, and $\delta \text{H}\beta = 0.91 \pm 0.03$) and the aromatic signals of the A ring which were found to be within a 4 Å distance to the ketone, especially position 6 ($\delta = 0.65 \pm 0.19$). Clearly the protons, which were the closest to the ketone function (within the 4 Å distance), showed greater differences in chemical shift between *cis* and *trans* isomers. These chemical shift differences result from anisotropic shielding effects introduced through overall perturbation of the 3-dimensional structure. The shielding effect can be induced by the ketone CO, the α - β unsaturation, and be modulated by the shielding zone of the aromatic rings [26]. Surprisingly, the percentage of *cis* isomer after more than a week of daylight exposure, was > 50% in DMSO- d_6 solution as exemplified with LicAF2 (Fig. 6), but also observed for LicAF1 and LicA (Supplementary Material, S6 and S10). Thus, the 1D ^1H NMR results confirmed that the two compounds detected in each LC-MS chromatogram of the LicAF sample are *cis* and *trans* isomers (Fig. 2), and indicate that photoinduced isomerization of retrochalcone solutions occurs spontaneously when unprotected from light and left at room temperature. Interestingly, the reverse phase LC separation of both *cis* and *trans* isomers, as seen with LicAF1-2, was not observed for LicA despite the different elution methods and selected stationary phases (C-18, C-8, Phenyl and Cyano). Nevertheless, both *cis* and *trans* isomers are clearly observable by NMR when the samples are diluted in DMSO- d_6 (Supplementary Material S6). Based on the results obtained herein, one can deduce that *trans-to-cis* photoisomerization will most likely occur for other licorice retrochalcones (i.e. licochalcone B, echinatin, Licochalcone C). However, additional extensive studies would be necessary to probe kinetic details, mechanism, conditions (nature of the solvents, temperature), and photo-physical aspects (quantum yield determinations and nature of excited state multiplicities) of licorice retrochalcone photoisomerization.

4. Conclusions

The structures of two LicA derivatives formed by oxidation (LicAF1) and reduction (LicAF2) of the aliphatic side chain of LicA were described. Both isolated compounds represented minor constituents of *G. inflata* crude extract, accounting for < 0.1% w/w. The isolation and structural characterization of LicAF1 are reported herein for the first time, whereas the structure of LicAF2 has been already disclosed as a product of the catalyzed rearrangement of LicA [17]. Additionally, the structural elucidation work presented herein, enabled the concomitant identification of both *cis* and *trans* LicAF1 as LicA phase I metabolites produced by human liver microsomes [27]. In the initial crude extract, LicA is the most abundant compound, representing almost 8% w/w (data not shown). Processes relating to the drying, grinding, extraction and fractionation of the plant material could have potentially generated a series of LicA by-products, similar to LicAFs. Such consideration also implies that other potentially new by-products could be found in the plant materials. Ultimately, all the minor LicA derivatives, usually found in the baseline of the chromatographic profiles, are part of the static residual complexity of the crude extract, and contribute to the pool of characteristic *G. inflata* retrochalcones.

It has been observed, herein, that these compounds spontaneously isomerize in solution when exposed to ambient daylight for several hours/days. As demonstrated with both isolated LicAF1-LicAF2 and LicA, the ratio of *cis* to *trans* isomers was found to be > 1 in the deuterated NMR solvent, after several days of light exposure, suggesting that the produced *cis* isomer is structurally stable under the observed conditions [28]. This photoinduced *trans* to *cis* isomerization is a good example of dynamic residual complexity, which is the result of the chemical instability or reactivity of a test compound [29][30]. Such observations are quite remarkable in this case as it is generally accepted that *trans* chalcone are more stable than their *cis* isomer. Moreover, *cis* isomerization of licorice retrochalcone such as echinatin and LicA (MeOH solution at 0.5 mg/mL), has been reported to be “unreactive” after a week of exposure to ambient daylight [9], which could be explained by the difficulty to separate *cis* and *trans* isomer of LicA by reverse phase LC. However, we observed herein, through the analysis of the NMR spectra, that after 1-week of daylight exposure, both the newly described retrochalcones and LicA (DMSO-*d*₆ solution at 5.1 mg/mL), were converted to a mixture of *cis* and *trans* isomers, with the *cis* isomer predominating in the less concentrated LicAF solutions. Clearly, the photoisomerization mechanism of retrochalcones remains a relatively unexplored area, as opposed to the studies dedicated to the photo-cyclisation of 2'-hydroxchalcones leading to the formation of flavanone isomers [30][11], the photoisomerization of 2-hydroxychalcone related to the formation of a flavylum structure [31][32], and the photochemical transformation of various *para*-substituted chalcones [33]. Further studies will be necessary to evaluate the mechanistic processes and photo-dynamics of retrochalcone isomerization under various conditions (e.g., solvent, concentration, temperature).

The dynamic residual complexity of chalcone photoisomerization could lead to variations in biological responses observed over time. In light of the diverse biological properties attributed to these retrochalcones [34], notably LicA and its derivatives [35][36][37][38][39], the photoisomerization studies conducted under bio-assays conditions [30], deserves consideration, as the variable ratio of *cis* and *trans* isomers may exert an effect on the biological response over time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Two dihydrobenzofuran derivatives of licochalcone A (LicA) were isolated as minor constituents from *G. inflata* crude extract
- Compound **1, LicAF1**, is described herein for the first time
- Compound **2, LicAF2**, has been previously produced by Lewis acid catalyzed rearrangement of LicA
- Both compounds and LicA undergo a photo-induced *trans/cis* isomerization
- The ^1H chemical shifts of the *cis* isomers exhibit a consistent up-field shift compared to the corresponding *trans* isomers

A Retrochalcone biosynthetic route

B Licochalcone A retrochalcone congeners

Fig. 1.

Echinatin, licochalcone A (LicA) isomers and its dihydrobenzofuran derivatives. **A.**

Summary of the biosynthetic route leading to the production of echinatin, the first described retrochalcone [7][8][9]. During the biosynthesis, methoxylation occurs in position 2', while the adjacent keto group undergoes a reduction and dehydration, thereby leading to an inversion of the identified A- and B-rings. Hence, retrochalcones are characterized by a lack of oxygen functionalities in C-2'/C-6', and the presence of a methoxy group in position 2.

B. Licochalcone C, E, F and compound 2 are all licochalcone A isomers, here represented in the *trans* geometry. Licochalcone A isomers as well as compounds 1 and 2 share the same retrochalcone core structure corresponding to echinatin, (black carbon edges), but differ structurally by the type of aliphatic side chain attached to C-5. For compounds 1 and 2, this chain forms a dihydrofuran ring with the hydroxyl in C-4.

Fig. 2.

LC-MS chromatograms and MS/MS spectra of compounds 1 and 2. LC-MS Chromatograms (left side) and MS/MS spectra (right side) readily obtained after isolation of compounds **1** (A) and **2** (B). The MS/MS spectra were taken at 20eV in positive ionization mode. The extracted ion chromatograms (m/z 355 for compound **1**, and m/z 339 for compound **2**) displayed two peaks with the exact same MS/MS spectra, thereby suggesting the presence of two isomeric forms. The fragment ion at m/z 121 suggests a hydroxyphenyl moiety, whereas the loss of ketene (m/z 313 and 297, respectively) confirmed that both compounds and their isomeric forms are 2-methoxy or 2-hydroxychalcone, as previously described [25].

Fig. 3.

Comparison of the 1D ^1H NMR spectra of compounds **1** and **2** ($\text{DMSO}-d_6$, 900 MHz) revealed that both compounds share the same pattern of aromatic protons associated with the retrochalcone core. The main differences are related to a set of signals corresponding to the methyl protons. Compound **1** (**LicAF1**) has two sets of methyl groups, whereas compound **2** (**LicAF2**) has three sets, including one doublet having the same coupling constant ($J = 6.53$ Hz) as the quartet proton at 4.45

Fig. 4. Overlay HMBC and HSQC spectra for structural determination of compound **1** (**LicAF1**). The structural assignment on the overlay HMBC and HSQC spectra of (trans) compound **1** (**LicAF1**) is represented by color coded arrows. All the correlations observable for H-6 and H-3 are in green and red, respectively. The correlations related to the α - β unsaturated ketone unit are in dashed lines, and finally the correlation associated with the dihydrofuran moiety and the methoxy group are in blue. The bold bonds of the structure represent the observable COSY correlations. The same type of observations was detected for compound **2** (raw data are deposited in the Harvard Dataverse repository at doi:<http://dx.doi.org/10.7910/DVN/DFAVTE>).

Fig. 5.

^1H NMR spectrum of **LicAF1**, and chemical shift difference between *trans* & *cis* isomers.

3D representations of **LicAF1** optimized inside the MMS module of the PERCH software, ^1H NMR spectrum (Exp., 900 MHz, $\text{DMSO}-d_6$) and HiFSA simulated (Sim.) spectra obtained for both *trans* (green) and *cis* (red) isomers and showing the up-field shifts of proton resonances corresponding to the *cis* isomer. The ratio of isomers was calculated as 10.6/89.4 *cis/trans* for a sample protected from light prior to NMR analysis.

Fig. 6.

^1H NMR spectrum of **LicAF2**, kept in day light, and chemical shift difference between *trans* & *cis* isomers. (A) 3D representations of **LicAF2** optimized inside the MMS module of the PERCH software, ^1H NMR spectrum (Exp., 900 MHz, $\text{DMSO}-d_6$) and HiFSA simulated (Sim.) spectra obtained for both *trans* (green) and *cis* (red) isomers and showing the up-field shifts of proton resonances corresponding to the *cis* isomer. The ratio of isomers was calculated as 52.5/47.5 *cis/trans* for a sample unprotected from light a week prior to NMR analysis. (B) Calculated ^1H and ^{13}C chemical shifts (δ mean $\pm$ stdv) between the *trans* and *cis* isomers, for LicAFs and LicA. The results suggest a general anisotropic shielding effect for the ^1H occurring through the modification of the 3-dimensional structures. A deshielding effect was measured for the C=O, C- α and -6, whereas C-2, C-5 and to a lesser

extend C-4 were found to be more shielded in the *cis* isomer. The most important δ were measured for the C=O, CH- α , CH- β , and CH-6.

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Table 1
¹H and ¹³C NMR Spectroscopic Data of Compound 1 = LicAF1 (DMSO-*d*₆, 900MHz)

Position	mult.	LicAF1 (<i>trans</i>)		LicAF1 (<i>cis</i>)	
		δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
C=O			187.10		δ
1			116.35		δ
2 (OCH ₃)			159.79		158.48
3	1H	6.57, <i>s</i>	94.37	6.43, <i>s</i>	94.06
4 (O-)			161.94		161.84
5			130.52		128.87
6	1H	7.84, <i>s</i>	122.29	7.08, <i>s</i>	124.38
2',6'	2H	8.03, AA' type (0.19/2.71/8.60)*	131.37	7.73, AA' type (0.19/2.71/8.60)*	131.48
5',3'	2H	6.87, XX' type (0.19/2.46/8.60)*	115.79	6.74, XX' type (0.19/2.46/8.60)*	115.75
4'(-OH)			δ		δ
α	1H	7.72, <i>d</i> (15.54)	118.35	6.47, <i>d</i> (12.77)	124.56
β	1H	7.99, <i>d</i> (15.54)	137.94	7.03, <i>d</i> (12.77)	133.93
2''	1H	4.33, <i>dd</i> (4.61/7.55)*	94.07	4.18, <i>dd</i> (4.63/7.39)*	93.77
3''			42.39		42.03
4''a	1H	3.69, <i>dd</i> (7.55/-11.83)*		3.60, <i>dd</i> (7.39/-11.79)*	
4''b	1H	3.73, <i>dd</i> (4.61/-11.83)*	60.31	3.63, <i>dd</i> (4.63/-11.79)*	60.13
5'' & 6''(-CH ₃)	3H $\times$ 2	1.16, <i>s</i>	23.36	0.90, <i>s</i>	23.23
O-CH ₃	3H	3.85, <i>s</i>	56.61	3.71, <i>s</i>	56.25

* Calculated values obtained through full spin analysis using Perch NMR software, in order to obtain the exact coupling constant (see supplementary material).

δ_{No} ¹³C, and HMBC correlation observed.

Table 2
¹H and ¹³C NMR Spectroscopic Data of Compound 2 = LicAF2 (DMSO-*d*₆, 900MHz)

Position	mult.	LicAF2 (<i>trans</i>)		LicAF 2 (<i>cis</i>)	
		δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}
C=O			187.11		192.56
1			115.82		116.46
2 (OCH ₃)			160.00		157.89
3	1H	6.55, <i>s</i> (0.43 Hz) *	94.14	6.42, <i>s</i> (0.57 Hz) *	93.69
4 (O-)			161.84		157.87
5			130.39		128.46
6	1H	7.85, <i>s</i> (0.43 Hz) *	122.31	7.07, <i>s</i> (0.57 Hz) *	124.29
2', 6'	2H	8.03, AA' type (0.49/2.55/8.47) *	130.82	7.72, AA' type (0.15/2.53/8.59) *	131.08
5', 3'	2H	6.89, XX' type (0.49/2.16/8.47) *	115.36	6.75, XX' type (0.15/2.53/8.59) *	115.13
4'(-OH)			δ		161.85
α	1H	7.72, <i>d</i> (15.55)	117.87	6.47, <i>d</i> (12.55)	123.26
β	1H	7.99, <i>d</i> (15.55)	137.87	7.05, <i>d</i> (12.55)	133.76
2''	1H	4.47, <i>q</i> (6.53)	89.81	4.30, <i>q</i> (6.53)	89.40
3''			42.61		42.50
4'' (-CH ₃)		1.32, <i>d</i> (6.53)	14.48	1.22, <i>d</i> (6.53)	14.53
5'' & 6'' (-CH ₃)	3H $\times$ 2	1.33, <i>s</i> 1.09, <i>s</i>	26.20 23.25	1.06, <i>s</i> 0.84, <i>s</i>	26.11 23.02
O-CH ₃	3H	3.83, <i>s</i>	56.03	3.70, <i>s</i>	55.75

* Calculated values obtained through full spin analysis using Perch NMR software, in order to obtain the exact coupling constant (see supplementary material).

δ_{No} ¹³C, and HMBC correlation observed.