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Dynamic Residual Complexity of the Isoliquiritigenin−Liquiritigenin Interconversion During Bioassay†

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Supporting Information

ABSTRACT: Bioactive components in food plants can undergo dynamic processes that involve multiple chemical species. For example, 2′-hydroxychalcones can readily isomerize into flavanones. Although chemically well documented, this reaction has barely been explored in the context of cell-based assays. The present time-resolved study fills this gap by investigating the isomerization of isoliquiritigenin (a 2′-hydroxychalcone) and liquiritigenin (a flavanone) in two culture media (Dulbecco's modified eagle medium and Roswell Park Memorial Institute medium) with and without MCF-7 cells, using high-performance liquid chromatography−diode array detector−electrospray ionization/atmospheric pressure chemical ionization−mass spectrometry for analysis. Both compounds were isomerized and epimerized under all investigated biological conditions, leading to mixtures of isoliquiritigenin and *R*/*S*-liquiritigenin, with 19.6% *R* enantiomeric excess. Consequently, all three species can potentially modulate the biological responses. This exemplifies dynamic residual complexity and demonstrates how both nonchiral reactions and enantiomeric discrimination can occur in bioassay media, with or without cells. The findings highlight the importance of controlling in situ chemical reactivity, influenced by biological systems when evaluating the mode of action of bioactives.

KEYWORDS: *bioassay, epimerization, isoliquiritigenin, isomerization, liquiritigenin, residual complexity*

INTRODUCTION

The chalcone isoliquiritigenin (2′,4,4′-trihydroxychalcone) is biosynthetically and chemically related to the flavanone liquiritigenin (4′,7-dihydroxyflavanone). Both of them are found in numerous edible plant species primarily belonging to the Fabaceae[1−3] or Amaryllidaceae families,[4] but they are also found in the Asparagaceae[5] and Bignonaceae[6] families. Despite their wide distribution, these two polyphenols have been mainly studied as bioactive constituents of *Glycyrrhiza* species (licorice, Fabaceae).[7,8] From a biosynthetic perspective, isoliquiritigenin (LigC for liquiritigenin chalcone; see discussion on nomenclature in the Supporting Information, S5) is not only the precursor and isomer of liquiritigenin (LigF for liquiritigenin flavanone) but also of many other flavonoids, formed as part of the phenylpropanoid pathway.[9] At the early stage of flavonoid biosynthesis, chalcone isomerase (CHI) promotes the conversion of 2′-hydroxychalcones, such as LigC, into stereochemically defined 2-*S* flavanones. During the enzymatic isomerization, the ionized chalcone is folded into a chiral conformation through interaction with the amino acid side chains in the CHI active site, which ensures high enantioselectivity of the flavanone production.[10,11] From a chemical perspective, the isomerization of 2′-hydroxychalcones has been described as occurring spontaneously in protic solvents to form racemic flavanones. The chemical mechanism sustaining the isomerization of 2′-hydroxychalcones, in solvents and nonbiological media, has been studied since 1975 and has already received considerable attention.[12−19] The cyclization of 2′-hydroxychalcones occurs through the initial deprotonation of the 2′-hydroxyl function and the subsequent formation of a 2′-oxyanion for the intramolecular Michael addition on the α-β unsaturated double bond. This mechanism depends on the pH of the aqueous solution and has been demonstrated to be affected by the nature of the α substituent of the chalcone (Figure 1).[12,13] At neutral and lower acidic pH, 2′-hydroxychalcones are unstable and undergo rapid cyclization into flavanones.[14,15] Under basic conditions, the flavanone isomers can undergo a reversible opening of their C ring leading to their corresponding chalcones.[16,17] In aqueous solution, this interconversion between chalcone and flavanone results in the racemization of optically active flavanones.[16,18] Representing a thermodynamic reaction, the rates of isomerization and racemization are influenced by temperature.[15,19] Thus, LigC and LigF are not only biosynthetically related but also chemically interchangeable.

Interestingly, the chemical conditions promoting this isomerization closely match the conditions of cell-based assays, where the cells are cultured in aqueous medium (protic solvent), at buffered and physiological pH (around 7.4), and at a slightly elevated temperature of 37 °C. Under these "biological" conditions, isomerization of 2′-hydroxychalcones such as LigC is very likely to occur. Consequently, it is reasonable to

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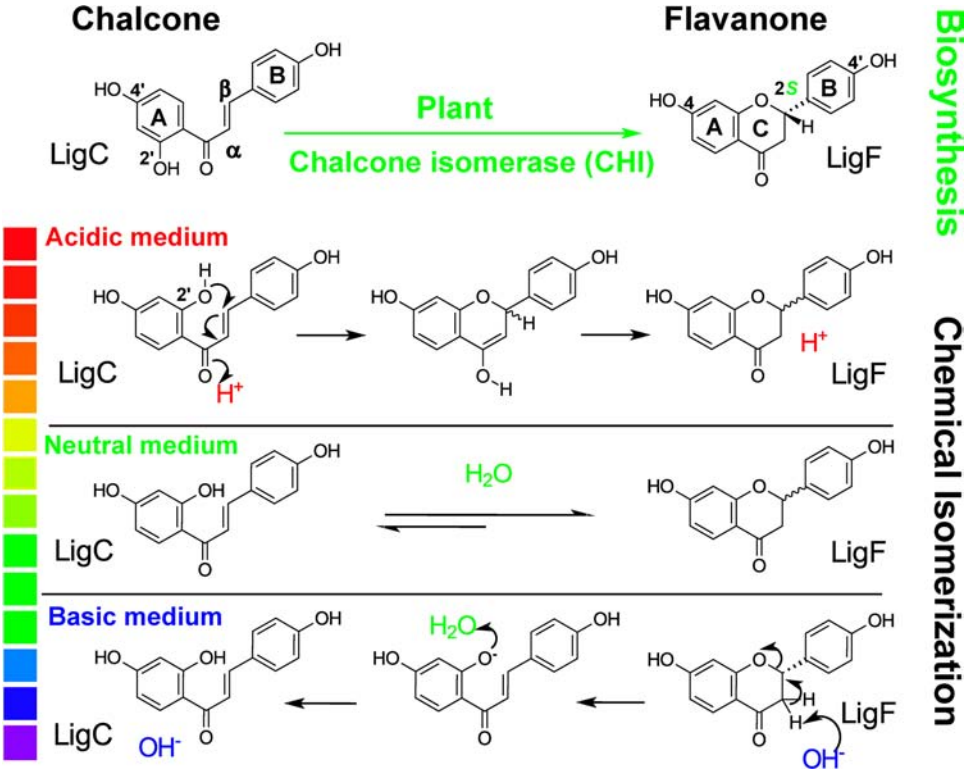

Figure 1. Overview of the chalcone−flavanone isomerization under biosynthetic and chemical conditions. Flavanone biosynthesis involves stereochemically defined isomerization of 2′-hydroxychalcones catalyzed by CHI. Nonenzymatic isomerization of 2′-hydroxychalcones−flavanones depends on the pH of the medium. In acidic solution, 2′-hydroxychalcones such as LigC fully isomerize into flavanones such as LigF. Under basic conditions, flavanones are unstable and produce 2′-hydroxychalcone through opening of the C ring. In neutral conditions, such as in physiological environments or cell culture media, the isomerization reactions occur in both directions, establishing a pH-dependent equilibrium of all potential species.

hypothesize that LigF is produced in the culture medium during bioassays and potentially contributes to the biological response. Such a LigC−LigF interconversion process would exemplify dynamic residual complexity (DRC), which is the result of the chemical instability or reactivity of a test compound and can explain variations in biological responses that are observed over time.[20]

This basic hypothesis is supported by previous studies that have shown that LigC and LigF have closely related biological activities. For example, both compounds have been reported to exhibit estrogenic,[21−23] anti-inflammatory,[24,25] anti-cancer,[6,26−39] hepato-,[40,41] and neuro-protective[42,43] properties (Supporting Information, S2). In cases where the biological effects of both LigC and LigF have been directly compared side-by-side, LigC has been shown to exhibit better activity than LigF.[6,21,25] In an effort to define their phytoestrogen potential, Miksicek compared the estrogenic potency of LigC and LigF, under identical experimental conditions using a 1 μM final concentration, and found that LigC displayed better estrogenic properties than LigF in competitive estrogen receptor (ER) binding and chloramphenicol acetyltransferase (CAT) enzyme assays on transfected HeLa and COS-7 cells.[21] In two different studies, LigF has been reported to be a selective ERβ agonist on transfected U2OS and HeLa cells,[22] while LigC exhibited agonist activities on both ERs (α/β) associated with a modulation of MCF-7 cells growth through ERα-dependent pathways and in a dose-dependent fashion.[23] Recently, Feldman et al. demonstrated that LigC exhibited better antibacterial activity than LigF against various oral pathogens and also showed better anti-inflammatory property through the inhibition of the NF-κB pathway.[25] However, the majority of the cell-based studies reported up to now have evaluated the biological properties of either LigC or LigF independently, rather than in parallel. This limits the confidence with which we can compare the biological properties of the two compounds by reference to the literature. While, Boumendjel et al. have highlighted the importance of the possible isomerization during cell-based assays with regard to the anticancer potential of chalcones,[44] this reaction has not been monitored in situ and confirmed to occur during cell-based assays.

Herein, we report on the evaluation and characterization of the isomerization of LigC and LigF at 37 °C, pH ∼7.4, in two widely used mammalian cell culture media, DMEM/F12 (Dulbecco's modified eagle medium), and RPMI 1640 (Roswell Park Memorial Institute medium). The study was performed both in the presence and absence of cultured MCF-7 cells, each over the period of 4 days. The MCF-7 cell line employed in the studies is an epithelial cancer cell line derived from a breast adenocarcinoma and represents one of the most commonly used ER positive breast cancer lines in biomedical research. Both LigF and LigC have displayed estrogenic activity in MCF-7 cells.[21−23] Considering that the chirality of some flavonoids (e.g., flavanones, flavanols, and isoflavans) is rarely addressed in biological experiments, the present study aimed at defining the stereochemical stability of isolated *S*-LigF under the chosen bioassay conditions. For this purpose, time-resolved studies of the dynamic isomerization of LigC and LigF were monitored by high-performance liquid chromatography

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(HPLC) with a photodiode array and dual ion source−mass spectrometry (DUIS-MS) under chiral and nonchiral conditions using a parallel protocol with Chiralpak IA and Shimadzu C18 columns, respectively.

One objective of this study was to probe the influence of bioassay conditions including the culture media (enriched buffered aqueous media), temperature, time of incubation, and presence of cells on the isomerization and racemization of chalcones and flavanones. Another goal was to demonstrate the occurrence of biochemical discrimination between LigF enantiomers by comparison of experiments in media with and without the presence of cultured cells. The experiments cover several aspects ranging from the analysis of dynamic isomerization to the evaluation of LigF epimerization under cell-based bioassay conditions. Overall, the study was designed to gain a better understanding of the inevitable chemical processes that underlie any biological test result when dealing with chemically unstable 2′-hydroxychalcones and their flavanone isomers.

MATERIALS AND METHODS

General Experimental Procedures. The following instruments were used to obtain physical data: circular dichroism (CD) spectra were acquired on a Jasco J 715 polarimeter (l = 1 cm); HPLC and LC-MS analyses were carried out on a Shimadzu model LCMS-2020 instrument (DUIS mode: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)), representing an integrated system with a diode array detector (DAD; semimicro flowcell, Shimadzu SPD-M20A) on a Shimadzu C18 (4.6 mm × 5.0 mm, 5 μm, cat no. 220-91394-00) or a Diacel Chiralpak IA (4.6 mm × 250 mm, 5 μm) column. DUIS-MS acquisition parameters were set as follows: probe voltage positive ionization mode, 4.5 kV; negative ionization mode, −4.5 kV; detector voltage, 1.2 kV; nebulizing gas flow rate, 1.5 L/min; drying gas flow rate, 10 L/min; DL temperature, 300 °C; heater temperature, 400 °C. The autosampler temperature was set at 4 °C, and the column oven temperature was set at 40 °C. Postrun data analyses were done with the Shimadzu LabSolution software package. The Shimadzu C18 column was eluted at 0.6 mL/min with a gradient composed from H_2O/0.1% formic acid (A) and acetonitrile/0.1% formic acid (B) as follows: 5−57% B in 18 min, 57−98% B in 7 min, and hold at 98% B for 3 min. The Chiralpak IA column was eluted isocratically with 55% acetonitrile (0.1% formic acid) in a reverse phase mode or with an *n*-hexane/ethanol gradient from 85% to 70% *n*-hexane in a normal phase mode, both at a flow rate of 0.7 mL/min. Countercurrent separation (CCS) was carried out on a hydrodynamic centrifugal coil instrument (TBE-300B, Shanghai Tauto Biotech Co., Ltd.) integrated with the Cherry-One automated CCS system (Cherry Instruments, Chicago, IL). Samples were dried using a Thermo−Fischer Savant SC250 EXP speed vacuum equipped with a RVT4104 refrigerator vapor trap. An EchoTherm CO30 column chiller−heater (Torrey Pines Scientific, Inc., Carlsbad, CA) was used to perform the defined temperature experiments.

Reagents. All chemicals and reagents including dimethylsulfoxide (DMSO) and HPLC-grade solvents were obtained from Fisher Scientific (Hanover Park, IL) or Sigma−Aldrich (St. Louis, MO). All media for cell culture were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). The adsorbents HW-40 F and LH-20, used for LigC and LigF isolation, were purchased from Tosoh Bioscience (King of Prussia, PA) and Sigma−Aldrich, respectively. For the preparation of LC calibration curves, LigF and LigC reference standards were purchased from Indofine Chemical Company Inc. (Hillsborough, NJ) and ChromaDex (Irvine, CA), respectively. A reference standard of 4′-hydroxyflavanone was used as an internal standard for LC-MS analyses and was also obtained from Indofine.

Plant Material and Extraction. Dried roots of *G. uralensis* Fisch. ex DC. were purchased from a local supplier (Chicago, IL). The plant material (voucher code BC 624) was identified through a series of macroscopic, and microscopic analyses as well as DNA authentication, comparing it to a voucher specimen from the Field Museum (Chicago, IL, voucher no. 2174544).

The powdered roots (998 g) were exhaustively extracted by percolation with MeOH at room temperature (wt/vol ratio = 1/20). Freeze-drying yielded 269 g of crude extract, representing 27% w/w of the powdered roots. The extract was stored at −20 °C prior to any chemical or biological analysis. A major portion of the crude extract (242 g) was dissolved in water/MeOH (80/20 v/v) and submitted to successive liquid/liquid partition with solvents of increasing polarity to yield five partitions: petroleum ether (22 g), $CHCl_3$ (8.5 g), EtOAc (14.2 g), *n*-BuOH (27.5 g), and water (73 g).

Isolation and Purification of LigF and LigC. LigF and LigC were isolated from the $CHCl_3$ partition of *G. uralensis*, after two steps of medium- and low-pressure liquid chromatography (MPLC, LPLC) using HW-40 F (100% MeOH, 2.5 mL/min) and Sephadex LH-20 (100% MeOH, 1 mL/min). The final purification of LigC and LigF was achieved by CCS (Cherry-One coupled to Tauto 300 mL), using a HEMWat 0 (hexane/EtOAc/MeOH/Water, 5:5:5:5) solvent system with the lower phase being mobile. The rotation speed was 800 rpm, the flow rate was 2 mL/min, the injection volume was 3 mL, and the stationary phase retention S*f* was 0.5. The *K* values of LigF (40 mg yield) and LigC (11 mg yield) in HEMWat 0 were determined to be 0.78 and 1.33, according to the retention volume, under these conditions. Compound identity and purity were assessed by means of qHNMR and MS analyses (Supporting Information, S3 and S4). Chiral analysis of LigF (40 μM in ethanol) by CD polarimetry was in accordance with previously published data.[45,46] The enantiomeric purity of LigF was further determined by chiral chromatography with UV detection at 275 nm using an *n*-hexane/ethanol gradient from 85% to 70% *n*-hexane in normal phase mode on a Chiralpak IA column.

Isomerization Study in Cell-Free Culture Media. Solutions of LigC and LigF were prepared in DMSO freshly and immediately prior to their dilution in both DMEM and RPMI (final concentrations 3 mM, 100 μM, and 5 μM), which were supplemented with 1% glutamax-1, 1% nonessential amino acids (NEAAs), and 0.05% insulin. DMEM and RPMI were additionally supplemented with 10% and 5% heat-inactivated FBS, respectively. DMSO alone diluted in RPMI and DMEM (0.1% v/v final) was used as a blank. Each of the LigC, LigF, and blank DMSO solutions was distributed in different vials and incubated at 37 °C. Samples diluted in phosphate-buffered serum (PBS) were used as controls. The influence of the temperature on the isomerization rate was also measured in PBS (100 μM final concentration) by keeping the samples in a thermostat at a programmed temperature (0, 15, 23, or 37 °C). Samples were collected every 2 h during a 12 h period and then twice a day for another 3 days. All samples were immediately partitioned with 3 volumes of EtOAc, and the organic layer was separated and dried in a speed vacuum concentrator for 2 h at 0 °C. At T_0, LigC and LigF were diluted in the media, which had been kept at the desired temperature, and were directly extracted with EtOAc without further incubation. The pH was checked for each time point using pH strips (Whatman, Panpeha, Sigma−Aldrich). Experiments were done in triplicate. Dried samples were diluted in 200 μL of HPLC-grade MeOH and centrifuged (2 min, 1400 rpm) prior to HPLC-DAD-MS analysis.

Cell Culture Conditions. The MCF-7 cell line was purchased from American Tissue Culture Collection (Manassas, VA). MCF-7 cells were grown in RPMI 1640 media containing 1% glutamax-1, 1% NEAAs, 0.05% insulin, and 5% heat-inactivated FBS. Two days prior to treating the cells, the medium was replaced with phenol red-free RPMI 1640 medium containing charcoal/dextran-stripped FBS with acetone-washed activated charcoal (100 mg/mL) at 4 °C for 30 min and centrifuged at 4000 rpm for 15 min at 4 °C. At 24 h prior to their treatment, cells were plated in 6-well plates (4.6×10^{-6} cells/mL) in phenol red-free RPMI 1640 supplemented with 5% heat-inactivated FBS, 1% glutamax-1, 1% NEAAs, and 0.05% insulin.

Isomerization Study in the Bioassay Conditions with MCF-7 Cells. MCF-7 cells were treated with either LigC or LigF at 5 μM final concentration (0.1% v/v DMSO) after 24 h incubation in the 6-well

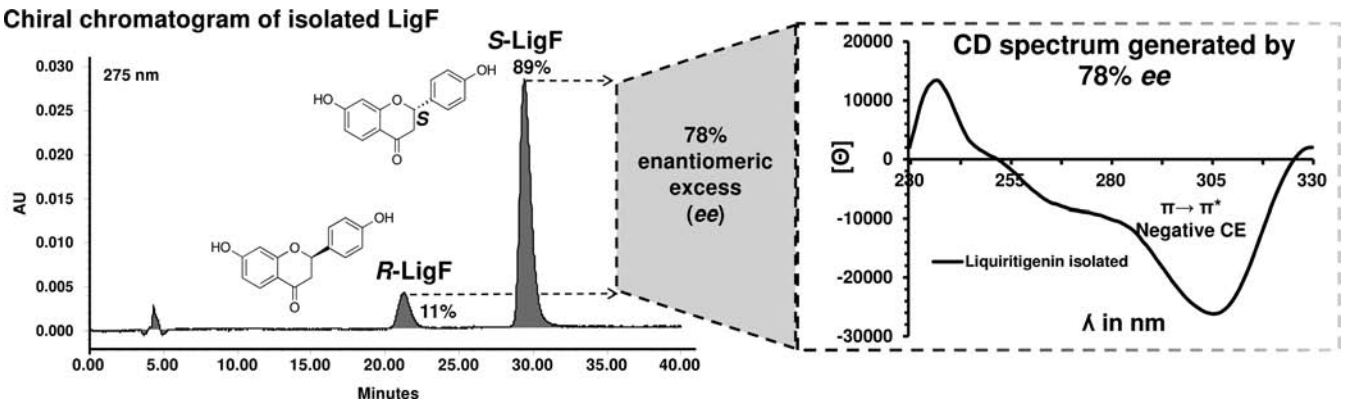

Figure 2. Absolute configuration and enantiomeric purity of isolated LigF. Chiral analysis of LigF, isolated from *G. uralensis*, was performed on a Chiralpak IA column with normal phase elution using a *n*-hexane/ethanol gradient. Enantiomers were detected at 275 nm. The *R*-LigF enantiomer was eluted at 21 min, and the *S*-LigF enantiomer was eluted at 29 min. Evidently, the measured CD spectrum of isolated LigF was generated by the ee of *S*-LigF (89% *S*-LigF − 11% *R*-LigF = 78% ee).

plates at 37 °C (5% CO_2). Under these experimental conditions, neither LigC nor LigF were cytotoxic. During the first 12 h, the treated cells and culture media were collected every 2 h and then once a day for the following 3 days. As described above, all samples were extracted by 3 volumes of EtOAc and dried in vacuo for 2 h at 0 °C. The cells were washed in PBS, treated with trypsin (5 min, 37 °C), and lysed in cold distilled water via two freezing (−80 °C)/thawing (room temperature) cycles. The cell content was extracted with EtOAc according to the same protocol used for the media. Experiments were done in duplicate. All dried samples were diluted in 200 μL of HPLC-grade MeOH, 10 μL of the internal standard solution (4′-hydroxyflavanone at 40 μg/mL) was added, and the mixture was centrifuged (2 min, 1400 rpm) prior to HPLC-DUIS-MS analysis.

Chromatographic Analysis by HPLC-DAD-MS. Samples were analyzed according to the LC conditions described above. The retention times (t_R) of LigF and LigC were 8.39 and 13.38 min, respectively, with the Shimadzu C18 column. With the Chiralpak IA column, *R*-LigF, *S*-LigF, and LigC exhibited t_R values of 6.71, 7.48, and 8.59 min, respectively. Selected ion monitoring (SIM) in both positive ($[M + H]^+$ m/z = 257) and negative ionization ($[M - H]^-$ m/z = 255) modes was chosen for MS data acquisition (see the General Experimental Procedures). For quantitative analysis, the area under the curve (AUC) values for both LigC and LigF were taken at 310 nm (maximum specific wavelength for both chalcones and flavanones) of the UV chromatograms, as well as determined from the MS chromatograms obtained in the negative ionization mode. In order to determine the relative abundance of each isomer at the different time points, the sum of LigF and LigC concentrations was normalized to 100%. The same normalization was applied to the determination of the ratio of each LigF enantiomer [100 = [*R*-LigF] + [*S*-LigF]] and to the evaluation of enantiomeric excess (ee). The regression equations used for the calculation of LigC and LigF concentrations (in mg/mL) were as follows: $AUC_{310\ nm} = 4 \times 10^6 \times [LigC] + 7070.5$, $r^2 = 0.9959$ and $AUC_{m/z=255} = 2 \times 10^8 \times [LigC] + 462\,929$, $r^2 = 0.9861$ for LigC; $AUC_{310\ nm} = 3 \times 10^6 \times [LigF] + 302.62$, $r^2 = 0.9999$ and $AUC_{m/z=255} = 1 \times 10^8 \times [LigF] + 276\,223$, $r^2 = 0.9945$ for LigF; where [LigF] or [LigC] are the concentrations of LigC or LigF in milligrams per milliliter (Supporting Information, S6).

Statistics. Quantitative data represent mean ± standard deviation of independent experiments. Statistical comparison of results was made using analysis by Student's *t* test. Differences were considered significant (*) for $p < 0.05$.

RESULTS

Characterization of Enantiomeric Purity of Liquiritigenin. LigC and LigF were isolated from the $CHCl_3$ partition of *G. uralensis* after two steps of MPLC and one final CCS step using the HEMWat 0 solvent system.[47] The identity and purity of both polyphenols were monitored by qHNMR and MS analysis (Supporting Information, S3 and S4). LigF stereochemistry and enantiomeric purity were defined successively by CD polarimetry and chiral chromatography. The CD spectra of flavanones with *S* configuration at C-2 exhibit a positive Cotton effect in the n→π* UV absorption band at 330−320 nm and a negative Cotton effect in the π→π* absorption band with the 270−290 nm maximum.[45] The CD spectrum obtained for LigF (40 μM, in ethanol) was consistent with previously reported data, confirming the *S* configuration at C-2 (Figure 2).[46] Additionally, in order to study the time-resolved racemization of *S*-LigF, its enantiomeric purity was analyzed by chiral chromatography. For this purpose, a Chiralpak IA column was used in normal phase mode with a gradient of *n*-hexane/ethanol, and the separated LigF enantiomers were detected at 275 nm. The chromatogram revealed that isolated LigF contained 11% *R*-enantiomer (t_R = 21 min) versus 89% *S*-enantiomer (t_R = 29 min). The enantiomeric identity and ratio were determined by CD polarimetry of each enantiomer and measurement of their AUCs at 275 nm.

Isomerization in the Culture Media without Cultured Cells. In a first set of analyses, the isomerization of LigC and LigF was characterized in the absence of cultured cells, thus excluding the potential influence of cellular absorption and metabolism. The isomerization reactions were studied in two widely used culture media, RPMI 1640 and DMEM/F12, over 4 days and at 37 °C. The data obtained in these culture media were compared to those acquired in PBS. Each culture medium, in contrast to the PBS, was supplemented with 5% (for RPMI) to 10% (for DMEM) heat-inactivated FBS, 1% glutamax-1, 1% NEAAs, and 0.05% insulin. Therefore, the supplemented media contained a series of chiral molecules mostly in the form of the FBS proteins and their constituent L-amino acids. Three different final concentrations (5 μM, 100 μM, and 3 mM) of either LigF or LigC were tested. At regular intervals, samples were taken and extracted with EtOAc to separate the flavonoids from the unwanted proteins and other medium constituents. At T_0, LigC and LigF were added to the media, which had been kept at 37 °C, and were directly extracted with EtOAc without further incubation. After evaporation and dissolution in MeOH, the samples were analyzed by chiral HPLC using detection at 310 nm. The AUCs of LigC and LigF were used to calculate their concentration and final ratio in the analyzed samples. No isomerization was observed to occur during the HPLC analysis,

as indicated by the UV-MS chromatogram of freshly prepared LigF and LigC solution in MeOH (Supporting Information, S4). During all the experiments, the pH of each medium remained around 7.4 (Figure 3). At 37 °C, the isomerization

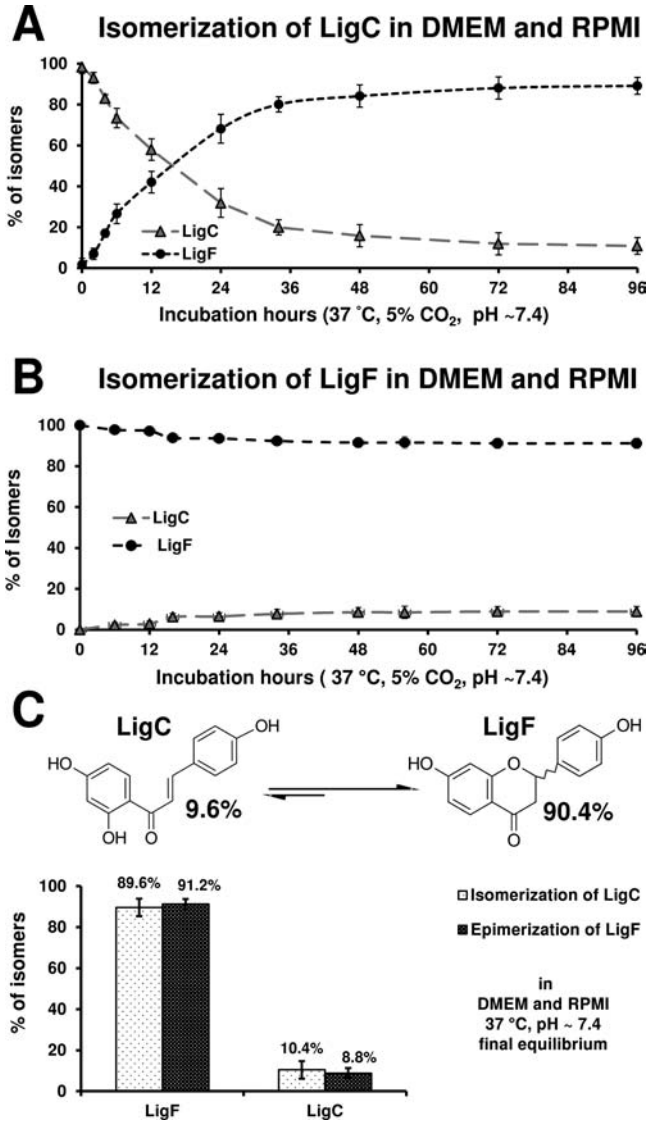

Figure 3. Time-resolved study of LigC and LigF isomerization in culture media without cells. Panels A and B represent the relative abundance of each isomer present in the media at 37 °C over a period of 4 days, leading to the final equilibrium of LigF and LigC (C). When the isomerization study started with LigC (A), a steady state was reached in 48 h and characterized by a ratio of 89.6 ± 4% LigF for 10.4 ± 4% LigC (C). When the isomerization study started with LigF (B), the steady state was reached in 10 h and characterized by a ratio of 91.2 ± 2.5% LigF to 8.8 ± 2.5% LigC. The relative abundance of each compound was calculated according to their concentration in both culture media. Experiments were done in triplicate. No significant differences were observed for the ratio of enantiomers calculated at the equilibrium of both reactions.

reaction of LigC began immediately and reached a steady state after 48 h of incubation in all media, indicating that, under those conditions, the isomerization came to a low energy state. The final equilibrium, characterizing this isomerization in both culture media, was defined by a ratio of 10.4 ± 4.0% LigC to 89.6 ± 4.0% LigF. When the study was initiated with the flavanone, LigF, at 37 °C, the equilibrium was achieved within 10 h and defined by the same LigC/LigF ratio, confirming the reversible interconversion between the two compounds. Identical results were obtained regardless of media (DMEM, RPMI, PBS) and concentrations of LigF or LigC tested (data not shown).

At physiological pH, the isomerization reaction is a unimolecular reaction defined by either the intramolecular cyclization of the 2′-hydroxychalcone or by opening of the C ring of the flavanone. Both reactions are initiated by the presence of free protons in the aqueous media. Moreover, the observed chalcone−flavanone interconversion in the media emphasizes the dynamic instability of the flavanone ring. This observation strongly suggests the occurrence of an epimerization−racemization of the optically active *S*-LigF, following a Wessely−Moser rearrangement.[48] Interestingly, in both culture media, the isomerization of LigC did not lead to a racemic solution of LigF but to an enantiomeric mixture characterized by a ratio of 53.2 ± 1.7% *R*-LigF to 36.4 ± 0.9% *S*-LigF (*R*-LigF ee = 17.2 ± 4.6%) after 24 h at 37 °C (Figure 4A,D). In contrast, racemization of LigF was observed within 6 h of incubation. The epimerization reaction also favored the production of the *R*-LigF enantiomer, with an ee of 7.4 ± 2.3% observed after 10 h of incubation in both RPMI and DMEM media but not in PBS (Figure 4B,D and the Supporting Information, S8). This indicates that the chiral environment of the culture medium induces asymmetric ring closure of the flavanone, leading to the production of *R*-LigF in excess. Moreover, this asymmetric induction was more important during LigC isomerization than LigF epimerization. Under the conditions of the bioassay, where samples are highly diluted in the culture media, the cyclization of LigC and the epimerization of LigF can be defined as either a zero- or a first-order reaction, until both reactions slow down and reach the steady state (Supporting Information, S7). According to the rate constant (k_{eq}) for the equation of a zero-order reaction (Figure 4C, eqs 1 and 2), the LigF epimerization was about twice as fast as the LigC isomerization during the first 8 h of incubation. The half-life of the isomerization reaction of LigC, defined as the time needed to isomerize 50% LigC (or until only 50% LigC remained), was calculated as $t^{1}/_{2}$ = 16.50 h (Figure 4C, eq 2), whereas for LigF epimerization, $t^{1}/_{2}$ = 6.30 h represented the time needed for the racemization of 50% *S*-LigF (Figure 4C, eq 1). The isomerization of LigC was observed to be a slower reaction compared to the epimerization of LigF. As such, LigC isomerization could enable a better chiral interaction with the culture medium, thus leading to a greater ee. The enantiomeric interconversion of flavanones through the production of an intermediate chalcone has been already demonstrated to occur for other flavanones such as naringenin and its glycosides, especially during maturation of plant material.[49,50] The present study demonstrates that, for LigF, this interconversion also occurs during the cell-based assay. Considering the stereochemical instability of LigF, it will be difficult or in many instances practically impossible to evaluate the true biological activity of a defined pure LigF enantiomer under the conditions of cell-based assays at 37 °C, especially when the final results require 12−24 h of incubation.

Influence of Temperature. As the isomerization−racemization of LigC and LigF was shown to occur in the culture media at 37 °C, at stable and physiological pH, the next step was to evaluate the influence of temperature on these

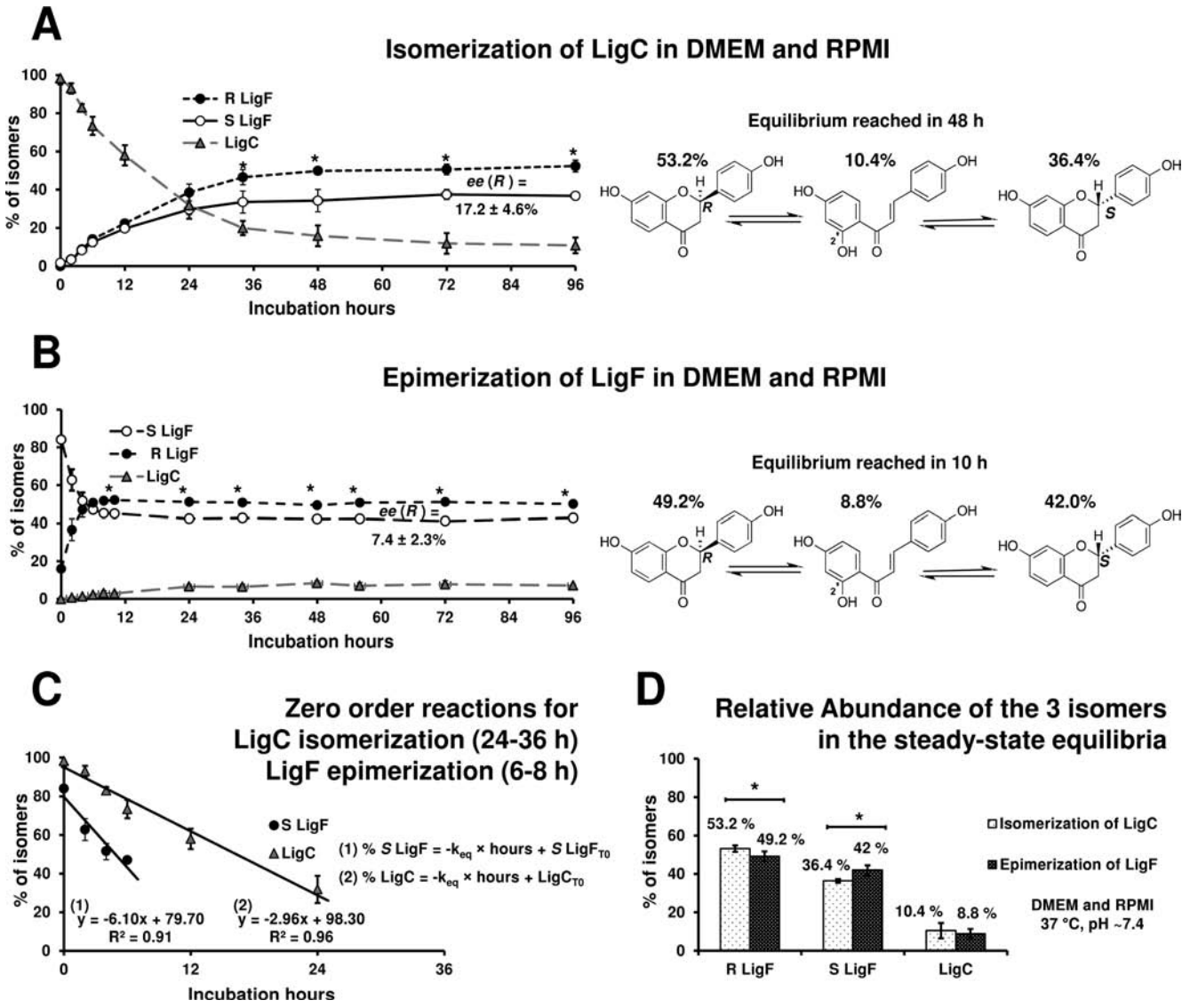

Figure 4. Stereochemical discrimination of LigF enantiomers during the isomerization of LigC and the epimerization of LigF in the culture media without cells. A and B represent the relative abundance of each isomer (LigC, *R*- and *S*-LigF) in the two media at 37 °C over a period of 4 days. When the isomerization study started with LigC (A), a significant ee of *R*-LigF at 17.2 ± 4.6% was observed and remained stable between 48 and 96 h. LigF, characterized by an enantiomeric ratio (er) of 89:11 (*S*/*R*) at T_0 (B), epimerized rapidly to yield a near racemic solution within 6 h of incubation. A slight ee of *R*-LigF at 7.4 ± 2.3% was observed after 10 h. The rate constant k_{eq} in the equations (C) demonstrate that LigF epimerization (k_{eq} = 6.10) was faster than LigC isomerization (k_{eq} = 2.96). The histogram D compares the relative abundance of *R*-LigF, *S*-LigF, and LigC at the reaction equilibrium. Experiments were done in triplicate.

reactions. Therefore, the reaction characteristics were studied at three additional temperatures (0−4, 15, and 23 °C) using PBS at pH ∼7.4 (Figure 5). As expected, the isomerization and racemization rates of both compounds were reduced at 23 °C and more so at 15 °C, compared to 37 °C. A steady isomerization state was reached after 96 h when conducting the experiments at these temperatures. A racemic solution of LigF was obtained after 24 h at 23 °C, compared to only 6 h at 37 °C (Figure 5B). However, at temperatures between 0 and 4 °C, no isomerization or racemization was observed. At physiological pH ∼7.4, in aqueous solution, in accordance with thermodynamic laws (Arrhenius equation), thermal energy can be regarded as the principal factor affecting both isomerization and racemization rates.[15,19] From a practical perspective, these results indicate that the bioactivity of single LigF enantiomers could potentially be evaluated if the experiments are performed at 4 °C or in less than 10 h at 15 °C.

In conclusion, this time-resolved study carried out under bioassay conditions without cells allowed a detailed characterization of the isomerization of LigC, which led to a final equilibrium of three species defined by 10.4 ± 4.0% LigC, 53.2 ± 1.7% *R*-LigF, and 36.4 ± 9% *S*-LigF after 2 days of incubation at 37 °C and pH ∼7.4 (Figure 4A). Epimerization of LigF led to a steady-state equilibrium after 10 h of incubation, characterized by a final composition of 8.8 ± 2.5% LigC, 49.2 ± 1.0% *R*-LigF, and 42 ± 0.5% *S*-LigF (Figure 4B). Finally, when starting with either pure LigF or LigC, three chemical entities were formed within 6 h of incubation. Their respective quantities were shown to change as a function of time and temperature.

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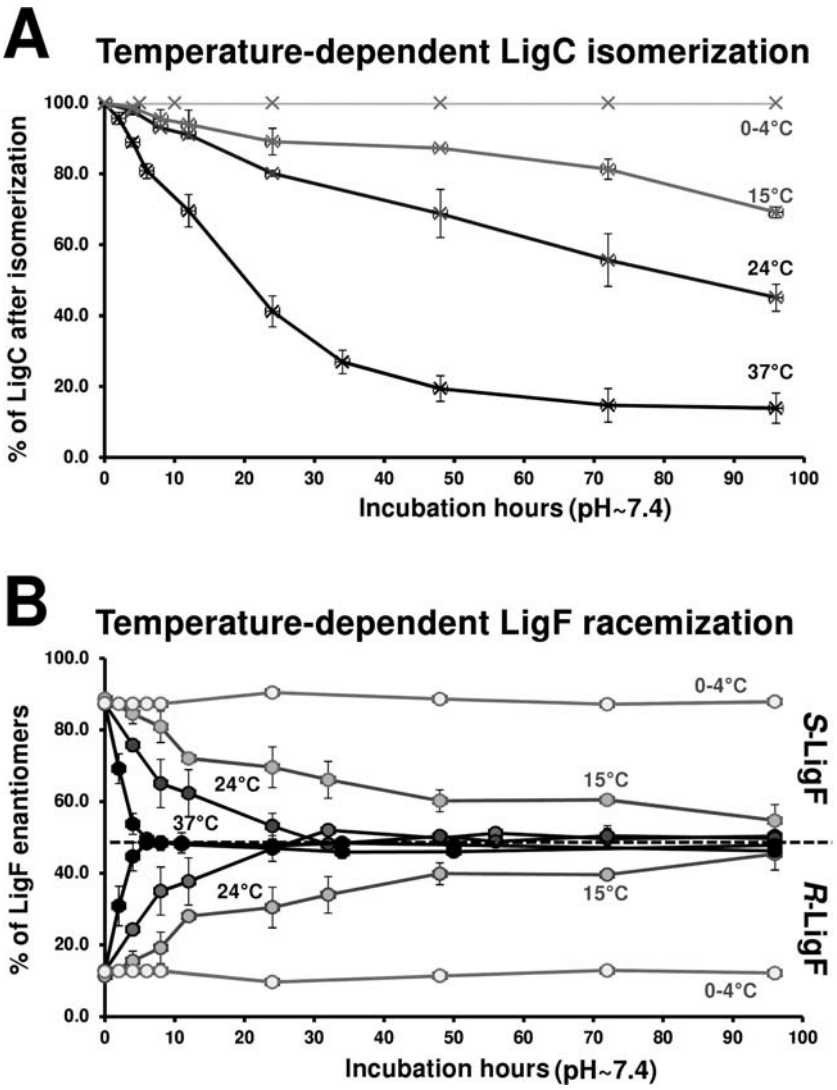

Figure 5. Temperature influence on the rates of isomerization and racemization. The relative abundance of LigC (A) and LigF enantiomers (B) is represented at different time points and for the four temperatures (0, 15, 24, and 37 °C), in PBS at pH ~7.4 (100 μM final concentration). Experiments were done in triplicate. At 0−4 °C, no isomerization of LigC (A) or racemization of LigF (B) was observed. However, at 15 °C, LigF racemization (B) was reached after 4 days of incubation, compared to 6 h at 37 °C. In an aqueous solution of physiologic pH, the rates of LigC isomerization (A) and LigF racemization (B) increased with rising temperatures.

Isomerization and Epimerization in the Presence of MCF-7 Cells. The second major aim was to determine whether the isomerization and epimerization reactions were affected by cellular absorption and metabolism. For this purpose, a time-resolved study was conducted in the presence of cultured MCF-7 cells, following the same incubation conditions previously described. It was hypothesized that a regular cellular uptake of LigC and LigF from the culture medium would affect the isomerization rate. The MCF-7 cells were treated with 5 μM of either LigC or LigF and incubated at 37 °C, with 5% CO_2, and over a period of 4 days. At this concentration, neither compound was cytotoxic. Samples from both the cells and the medium were taken at regular intervals, every 2 h during the first 12 h and then once a day until 4 days. At each time point, the extracts of the medium (RPMI 1640) and the intracellular contents were analyzed by DUIS LC-MS using SIM detection (m/z = 255) in negative ionization mode. The concentrations of LigC and *R*/*S*-LigF were determined for each sample using a standard calibration curve for each compound.

The decrease in LigC concentration in RPMI was more pronounced than that of LigF, indicating that freshly introduced LigC gets absorbed more rapidly by the cells (Figure 6A,B). This observation can only be partially explained by the difference in polarity/lipophilicity between the two compounds. Because of its $\alpha-\beta$ unsaturation, the chalcone tends to be more lipophilic than its flavanone counterparts, enhancing its passage through the cell membrane. Regardless of whether LigF or LigC were independently administered to the test system, the MCF-7 cells absorbed more than 50% of each compound within 24 h. Most of the cellular absorption occurred between 10 and 48 h of incubation, as indicated by the analysis of the cellular content (Figure 6C,D). During the course of the entire experiment, concurrent isomerization of LigC and epimerization of LigF were observed. The relative contents of LigC and its isomer, LigF, calculated from their respective concentration in RPMI, were identical during the first 10 h of incubation with or without cultured cells (Figure 6E). These percentages were clearly different from 10 to 48 h, indicating that the isomerization kinetics of LigC were affected

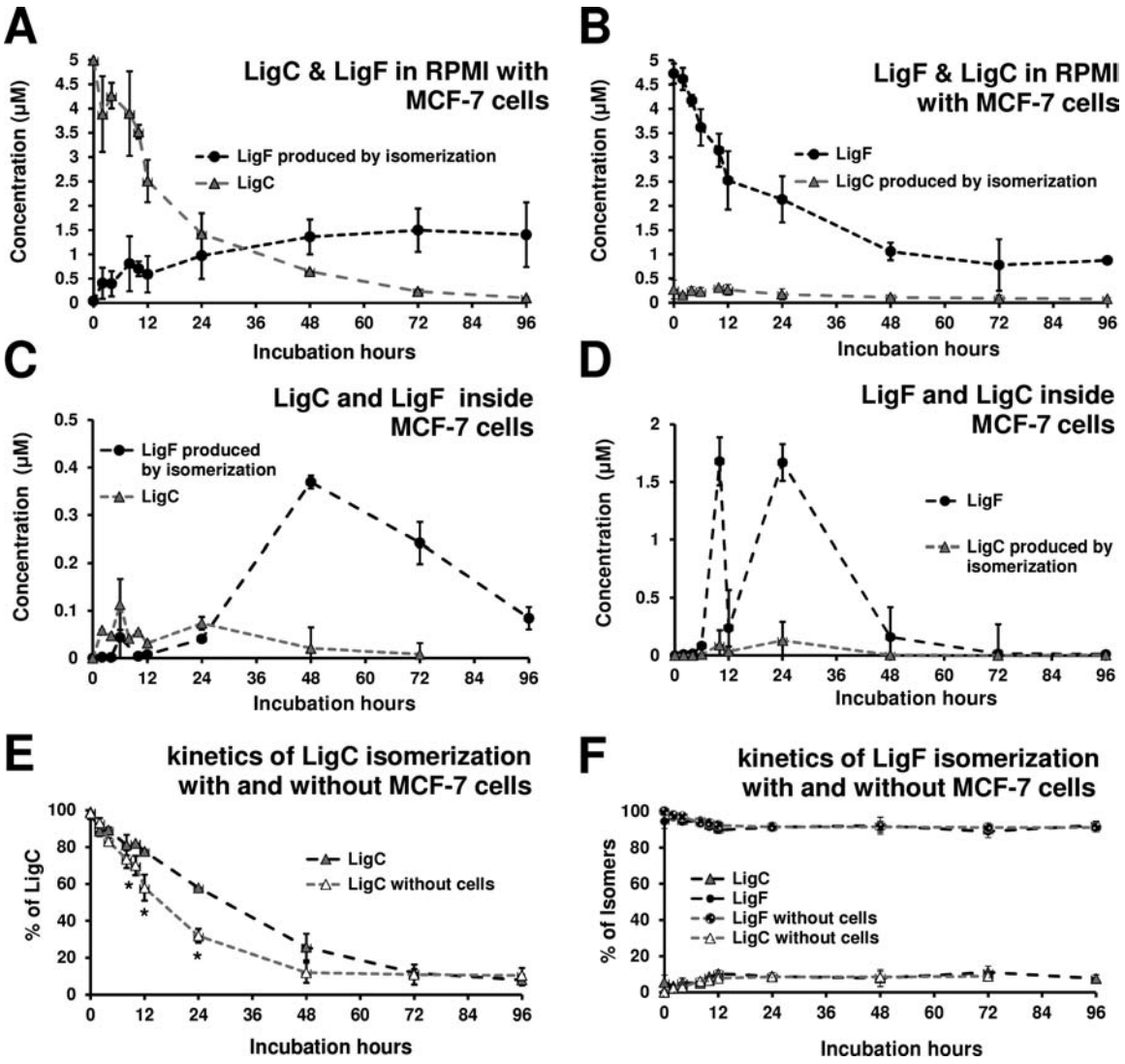

Figure 6. Isomerization of LigC and epimerization of LigF in the presence of MCF-7 cells. Panels A and C represent the concentration (μM) of LigC and its isomer produced in the RPMI medium and found inside the MCF-7 cells, respectively. Panels B and D represent the concentration (μM) of LigF and its isomer produced in the RPMI medium and found inside the MCF-7 cells, respectively. Cells were initially treated with 5 μM LigC or LigF. Data presented in the presence of cells were obtained from two independent and representative experiments. The isomerization of LigC occurred within 96 h of incubation in the presence of MCF-7 cells, as indicated by the increase in LigF concentration (A and C). However, the rate of isomerization was reduced from 10 to 48 h of incubation in the presence of cells (E), indicating that the cellular uptake slowed down the isomerization of LigC. The production of LigC from LigF occurred in RPMI medium with cells (B and D). The kinetics of LigF isomerization was not significantly different from those obtained without cells (D), indicating that the isomerization−epimerization reaction of LigF was not influenced by cellular uptake or metabolism.

by the cellular uptake of both LigC and LigF. For example, at 24 h, the LigC/LigF ratio produced by isomerization in the medium of cultured MCF-7 cells was 60:40, compared to a 35:65 ratio without cells. In this experiment, the cellular uptake/metabolism slowed down the isomerization of LigC between 10 and 48 h. After that, the cellular uptake seemed to be less pronounced, thus enabling the isomerization to become predominant and convert the remaining compounds in the medium. However, no significant differences were observed for the isomerization kinetics of LigF obtained with or without cells (Figure 6F). The racemization of LigF did also occur within 6 h of incubation in the presence of cultured cells (Figure 7A). Therefore, this reaction was not influenced by the cellular uptake, suggesting that LigF epimerization was faster than cellular absorption. Within 10 h, the stereochemical instability of LigF led to an *R*-LigF ee of 19.6 ± 4.0%, representing a value that was significantly higher compared to that obtained in the absence of cultured cells (ee = 7.4 ± 2.3%). This ee did not change within 48 h (Figure 7B). The same type of ee was observed between 10 and 48 h, regardless of whether the cells were treated with 5 μM LigC or LigF. Consequently, the *R*-LigF ee observed in RPMI in the presence of MCF-7 cells indicated that *S*-LigF was preferably absorbed by the cells, leading to an increased concentration of *R*-LigF in the medium (Figure 7C). The consideration of stereochemical instability of LigF and biological discrimination of its enantiomers could offer new insights in the interpretation of future bioassay results.

Isomerization of LigC and LigF occurs in the presence of cells and leads to a significant production of both isomers in the culture media but also inside the cells. The isomerization rate of LigC was altered by the cellular uptake but could also be

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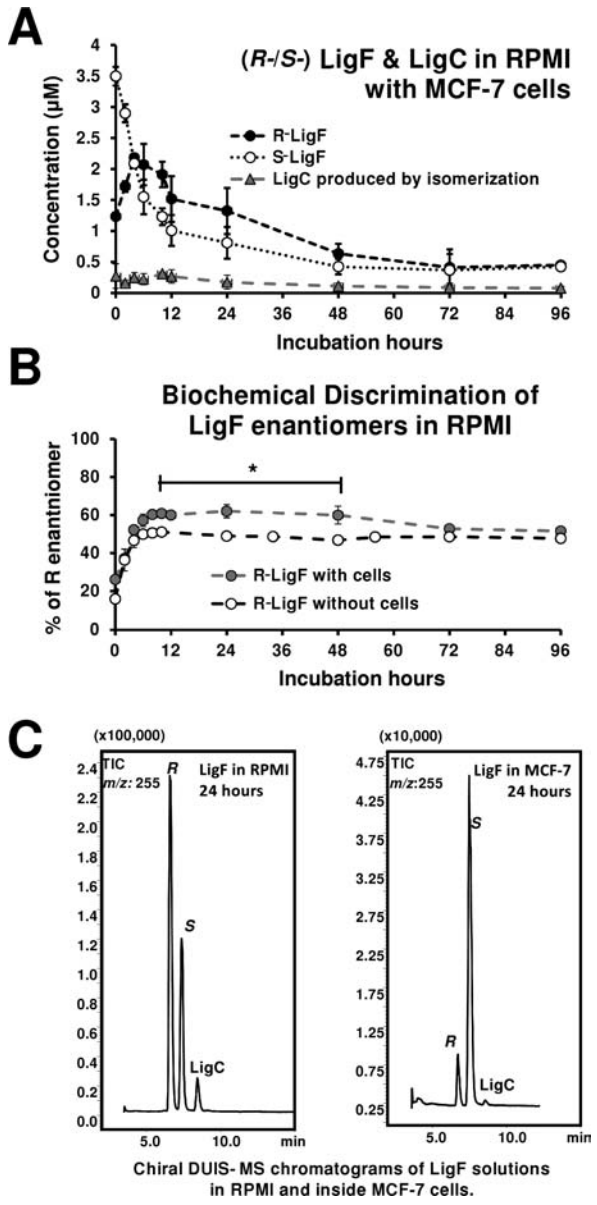

Figure 7. Biological discrimination of LigF enantiomers in RPMI in the presence of MCF-7 cells. Panel A presents the concentration (μM) of *R*- and *S*-LigF and its isomer LigC produced in RPMI medium with MCF-7 cells, initially treated with 5 μM LigF (er = 89:11 *S*/*R*-LigF). Panel B represents the relative abundance of *R*-LigF enantiomer, normalized to 100% for the sum of both LigF enantiomers remaining in RPMI. Data presented were obtained from independent and representative experiments. A significant *R* ee was observed in the medium of MCF-7 cells between 10 and 48 h. The chiral LC-MS chromatograms (C) of the solutions extracted from RPMI medium and MCF-7 cells after 24 h illustrate the *R*-LigF ee observed in RPMI, compared to the *S*-LigF ee determined inside the cells.

affected by the cellular density of the medium, the metabolic status of the cultured cells, and the type of investigated cell line. However, according to the present results, it is more likely that the epimerization of LigF will not be affected by these cellular factors but will rather occur rapidly in any bioassay conducted at 37 °C. Finally, because the three chemical entities are produced within 6 h of incubation, in the culture media, the administered compound no longer represents a pure agent, but is a residually complex mixture. As such, the resulting equilibrium cocktail could unfold more complex actions, for example, through synergistic, additive or antagonistic activities, to yield the finally observed biological test result. Accordingly, it does not appear to be feasible to truly separate the biological activity of LigC from those of its isomers in cell-based assays at 37 °C.

DISCUSSION

The present work studies the isomerization of LigC and the epimerization of LigF under cell-based assay conditions and highlights the complex dynamic equilibrium between 2′-hydroxychalcones and flavanones. The dynamic interconversion of LigF and LigC as well as the biochemical discrimination of LigF enantiomers in cell-based assay conditions is demonstrated for the first time. The evolution of the LigC/LigF ratio as well as *S*/*R*-LigF ee was shown to be a function of time, at a given temperature and physiological pH. The interconversion between LigC, LigF, and the LigF enantiomers represents an important example of DRC.[20,51] The three chemical entities are generated in the culture medium, simultaneously and in various proportions, and can modulate the ultimate biological response (Figure 8). Therefore, the observed bioactivity is a function of all the experimental conditions that affect the expression of the 2′-hydroxychalcone−flavanones equilibrium. These analyses shed a new light on the chemical conversion and complexity that underlies the biological response of what is widely considered due to "pure" LigC or LigF.

While epimerization of flavanones and isomerization of 2′-hydroxychalcones are considered relatively simple chemical reactions, it is important to note that these processes involve multiple unstable but potentially bioactive intermediates, which also must be present in the culture media, such as *cis*/*trans*-2′-hydroxychalcone or the chalconate anion.

Additionally, the present results highlight the importance of considering cell culture media as chiral environments that are able to chemically interact with compounds under investigation. During plant biosynthesis, the enantioselective production of *S*-flavanone is a consequence of the interaction between chalconate ions and the chiral amino acids of the CHI enzymatic core.[10,11] This reaction mechanism was recently modeled by Hintermann and Dittmer to chemically and selectively produce *S*-flavanone.[52] In a comparable way, the interaction of LigC with the amino acids and proteins of the culture media contribute to its asymmetric cyclization and lead to an ee. This reaction could occur through ion-pairing processes with chiral agents such as proteins and amino acids present in the culture media.

Many comparative biological studies have assigned LigC a greater activity than LigF when the respective compound was introduced in the bioassay (Supporting Information, S2). However, when taking into consideration the DRC beginning with LigC, it is entirely possible that the measured bioactivity might not be solely attributed to LigC but could be attributed to either combined (additive, synergistic, and/or antagonistic) effects between LigC and its isomers (*R*/*S*-LigF) and/or its metabolites produced during the experiments.[28,51] As the bioactivity of LigC can be linked to the bioactivity of its flavanone isomers, future studies are warranted that compare LigC and LigF side-by-side, so as to provide a better interpretation of their in vitro biological activities. For an even more accurate interpretation, it would be of interest to determine the LigC and LigF ratio in the culture medium at the time of the readings. However, if the biological experiments can

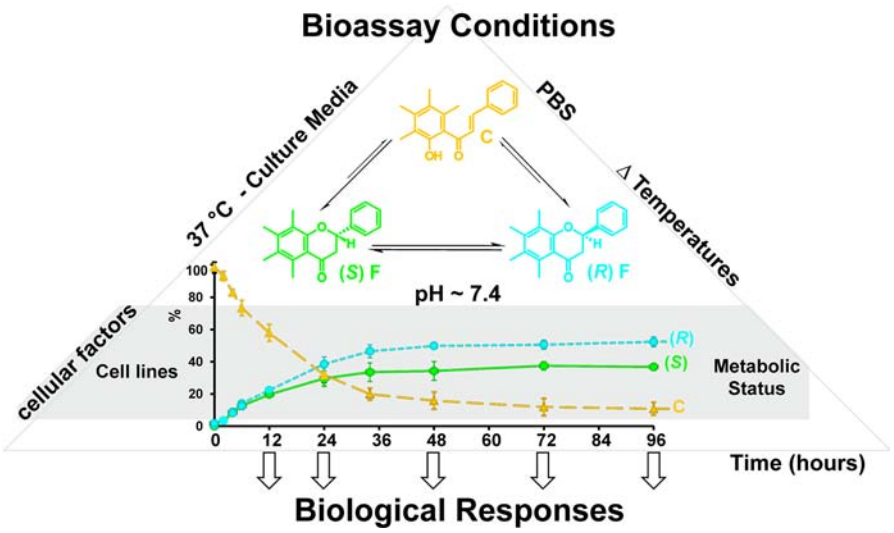

Figure 8. Actual expression of the DRC of 2′-hydroxychalcone−flavanone is a function of the individual bioassay conditions. Under cell-based assay conditions (37 °C, pH ∼7.4), the isomerization of 2′-hydroxychalcones (C) and the epimerization of flavanones (F) are principally a function of the incubation time and influenced by the chiral characteristic of the culture medium. The kinetics of isomerization and the ratio of flavanone enantiomers produced are affected by cellular factors, defined by the cell line chosen for the bioassay and its metabolic status. In PBS, isomerization and epimerization reactions are influenced by both temperature and incubation time. Consequently, the observed biological responses are convolutes of the bioassay conditions and the dynamic interconversion between 2′-hydroxychalcones and flavanones.

be performed at 0−4 °C, or at 15 °C in less than 10 h, or at 37 °C in less than 1 h, it should be possible to compare the biological activity of the individual pure species of LigC, *R*-LigF, and *S*-LigF. Under these bioassay conditions, observed effects such as receptor binding can be attributed with much higher confidence to the compound introduced initially.

The stereoselectivity of the biological environment in the cell-based assay for LigF enantiomers is another important consideration, as stereochemical discrimination affects not only the pharmacodynamic but also the pharmacokinetic (PK) parameters of LigF. This is illustrated by the study of Li et al., who demonstrated a predominant renal excretion of the *S*-LigF enantiomer after ingestion of herbal remedies.[53] Recently, Sayre et al. have shown that various PK parameters are clearly distinct for the enantiomers.[54] Although the PK analysis of LigF enantiomers has already received some attention, their potentially different pharmacodynamics have not been evaluated so far. Therefore, in future in vitro cell-based assays involving these compounds, the biochemical discrimination of LigF enantiomers should be considered.

Insights derived from the present study can likely be applied to other structurally related 2′-hydroxychalcones and their isomers. As suggested by previous chemical studies, the substitution pattern, especially in the A ring of 2′-hydroxychalcones and their flavanone isomers, will strongly influence their rate of isomerization and potential epimerization[15,19,55] but will also affect their cellular uptake.[56] Consequently, the kinetics of their isomerization in cell culture medium at 37 °C, as well as the final steady-state equilibrium of the reaction would be different for each individual 2′-hydroxychalcone−flavanone pair. Further studies comparing isomerization and racemization under bioassay conditions of various structurally different 2′-hydroxychalcones and flavanone isomers would allow establishment of structure isomerization relationships as a form of DRC and will be required to gain a better understanding of the biological activities of these related compounds.

The present results also emphasize the necessity of evaluating the chemical stability of compounds intended for biological evaluation in an aqueous medium at 37 °C. The choice of bioassay-like conditions for the study of chemical stability would provide a better identification and understanding of the lack or presence of RC of the actual bioactive compounds and could expedite discovery of the potential existence of prodrugs.

Because the interconversion between LigC−LigF and LigF enantiomers begins rapidly in buffered aqueous media at 37 °C, it is conceivable that the same reaction occurs during aqueous extraction of any plant material that contains 2′-hydroxychalcones, such as *Glycyrrhiza* sp. and *Medicago sativa*. The occurrence of isomerization during extraction can potentially affect both the relative ratio between 2′-hydroxychalcones and flavanones and the corresponding flavanone racemization or epimerization.[49,57] Finally, variation in the dynamic LigC−LigF isomerization−epimerization could also occur throughout plant life including biosynthesis and maturation, during the plant extraction process, as part of the in vitro bioassay, and during in vivo metabolism and elimination (gastrointestinal digestions,[57] liver metabolism, and renal excretion). The resulting various levels of RC make the correlation of chalcone−flavanone chemistry and biological activity an even more intricate task and requires a solid knowledge of the residually complex chalcone−flavanone equilibrium.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Publication series on residual complexity; a comparative table on the most frequently reported biological activities of LigF and LigC; the qHNMR and MS data obtained for isolated LigF and LigC; proposed naming scheme for chalcone−flavanone pairs; the UV and MS calibration curves used for the quantitation of these isomers in the different extracted solutions; the comparative LC-MS chromatograms obtained at different time points of the kinetic study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS USED

CCS, counter current separation; CD, circular dichroism; CHI, chalcone isomerase; DMEM, Dulbecco's modified eagle medium; DRC, dynamic residual complexity; DUIS, dual ion source (ESI and APCI); ee, enantiomeric excess; er, enantiomeric ratio; ER, estrogen receptor; FBS, fetal bovine serum; k_{eq}, rate constant of the kinetics equation; LC, liquid chromatography; LigC, isoliquiritigenin; LigF, liquiritigenin; NEAAs, nonessential amino acids; PBS, phosphate-buffered serum; $t^{i}/_{2}$, half-life of the kinetics equation; RC, residual complexity; RPMI, Roswell Park Memorial Institute medium

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