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Chalcone-Flavanone Equilibrium in the Isomeric Mixture: 2',3,4,4',6'-Pentahydroxychalcone and Eriodictyol from *Stevia lucida*

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Abstract

From the leaves and stems of *Stevia lucida* Lagasca (Asteraceae), an equilibrium chalcone-flavanone isomeric mixture, composed of 2',3,4,4',6'-pentahydroxychalcone (**8**) (eriodictyol-chalcone) and eriodictyol (**9**) [ratio **8**/**9** 5:3], was isolated as an isomorphic crystal. The mixture was structurally characterized by spectroscopic methods, including 1D- and 2D-NMR experiments. The presence of chalcones in the genus *Stevia* is reported here for the second time.

Keywords: Chalcones; Flavanones; Equilibrium Mixture; 2',3,4,4',6'-Pentahydroxychalcone; Eriodictyol; *Stevia*

Resumen

A partir de las hojas y tallos de *Stevia lucida* Lagasca (Asteraceae), se aisló una mezcla isomérica chalcona-flavanona en equilibrio, compuesta de 2',3,4,4',6'-pentahidroxichalcona (**8**) (eriodictyol-chalcona) y eriodictyol (**9**), como un cristal isomorfo en una proporción (**8**/**9**) 5:3. La mezcla se caracterizó estructuralmente mediante métodos espectroscópicos, incluidos experimentos de RMN 1D y 2D. La presencia de chalconas en el género *Stevia* se reporta aquí por segunda vez.

Palabras claves: chalconas; flavanonas; mezcla en equilibrio; 2',3,4,4',6' pentahidroxichalcona; eriodictyol; *Stevia*

Introduction

Flavonoids constitute one of the most important classes of naturally occurring phenols with interesting biological properties that demonstrate their great potential as treatment options to several human pathologies[1-4]. The scientific literature on the wide range of biological and pharmacological activities of flavonoids is extensive and review articles abound[5-12]. The so-called "minor flavonoids", among which stand out chalcones and flavanones, have received considerable attention in recent years, in the field of their biological activities[13-18].

It is currently well established that, from a biosynthetic perspective, flavonoids originate through the "*general phenylpropanoid pathway*", starting with *L*-phenylalanine (**1**) and then transforming it into *trans*-cinnamic acid (**2**) which it is hydroxylated to *p*-coumaric acid (**3**) and this evolves by esterification to *p*-coumaroyl-CoA (**4**). On some plants, the latter (**4**) may be hydroxylated in position 3 to give caffeoyl-CoA (**5**), which can also serve as substrate in the next biosynthetic stage[19-21] (Fig. 1). The known enzymes related to this pathway are phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), *p*-coumarate:CoA ligase (4CL), and 4-coumaroyl:CoA 3-hydroxylase (CC3H)[22]. Since the mid-1970s, substantial progress has been made on plant cell suspension cultures and these were a valuable source for the isolation and characterization of flavonoid enzymes[23,24]. The next biosynthetic stage carried out by the enzyme chalcone synthase (CHS)[25] focuses on the condensation of *p*-coumaryl-coenzyme-CoA (**4**) with three molecules of malonyl-CoA (**5**) [generated by the reaction of acetyl-CoA and CO_2 catalyzed by acetyl: CoA carboxylase (ACC)] giving the C-15 intermediate 2',4,4',6'-tetrahydroxychalcone (**6**). The most important stage in flavonoid biosynthesis is that corresponding to the conversion of chalcone (**6**) into the flavanone naringenin (**7**) since all the other flavonoids are generated from the latter. This stage is catalyzed by the chalcone isomerase (CHI) enzyme, which generates a chalcone-flavanone equilibrium[26].

Stevia lucida Lagasca (Asteraceae) is a widely distributed species in the Venezuelan Andean Region[27]. From a phytochemical point of view, we have studied this species for

more than three decades and have achieved to isolate and identify several steroids[28], flavonoids[28,29], diterpenes of the labdane[30] and *ent*-kaurane series including an interesting new *ent*-kaurenolido[31], longipinane sesquiterpenes[32], some eudesmanolides[33], and (+)-mellein[29], the first isocoumarin reported for the genus *Stevia*. 7β,9α-dihydroxyongipin-2-en-1-one, the most abundant sesquiterpene in *S. lucida*, has been an excellent substrate for chemical reactions[34,35], particularly Wagner-Meerwein rearrangements[36-38], which lead to new regrouped sesquiterpene skeletons. Other researchers have also investigated *S. lucida*, describing as components of this species a new labdane diterpenic acid[39] and several longipinene diesters[40].

Recently, we reported the isolation and characterization of the first three chalcones present in the genus *Stevia*[41]. Now, we describe a fourth chalcone, the 2',3,4,4',6'-pentahydroxy-chalcone (also called eriodictyol chalcone) (**8**) obtained from *Stevia lucida*, with the particularity that it is in its natural state in equilibrium with the corresponding flavanone eriodictyol (**9**). The mixture could not be separated by conventional chromatographic methods and it was characterized as such, through of a detailed spectral study that includes 1D and 2D-NMR techniques.

Experimental

General experimental procedures

Melting point was determined using a Fisher-Johns apparatus and it is uncorrected. UV spectra were obtained in a Perkin-Elmer spectrophotometer, Lambda 3B, using quartz cells with 1 cm thick and methanol (Merck-Uvasol) as solvent. IR measurement was obtained on a Perkin-Elmer FT-1725X spectrophotometer as KBr pellets. 1D and 2D NMR spectra in DMSO-d_6 were acquired using a Bruker-Avance DRX-400 instrument, operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Mass spectra were recorded on a *Hewlett-Packard* mass spectrometer, *model 5890* (70 eV). TLC were developed on 0.25 mm layers of silica gel PF 254 (Merck); spots were visualized using UV light (254 and 365 nm) and subsequently by spraying with a mixture *v/v* CH_3COOH-H_2O-H_2SO_4 (20:4:1) and then heating with air-flow at 100 °C for few minutes. VCC was performed with silica gel Merck 60 (63-200 μm, 70-230 mesh).

Plant material

Plant material (leaves and stems) was collected at "Páramo de la Negra, Municipio Rivas Dávila, Estado Mérida, Venezuela". Species was identified as *Stevia lucida* Lagasca by Eng. Juan Carmona Arzola, Department of Pharmacognosy and Organic Medicaments, Faculty of Pharmacy and Bioanalysis, University of Los Andes (ULA); a *voucher specimen* (JM Amaro-Luis & P. Chacón, No. 2332) was deposited at the Herbario MERF of this faculty.

*Extraction and purification of the equilibrium mixture of **8** and **9***

The dry uncrushed leaves and stems (≅ 4.0 kg) were exhaustively extracted with ethanol in a soxhlet. The obtained solu-

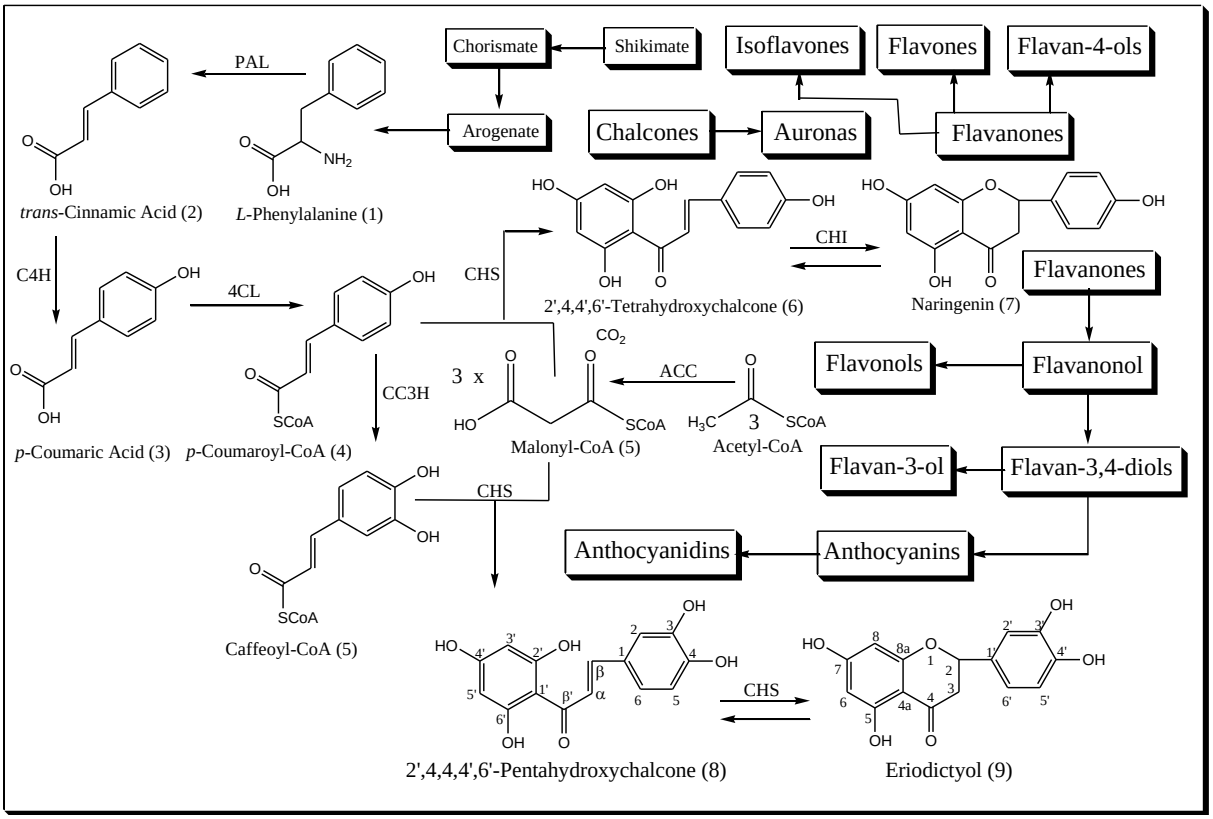

Fig. 1: Biosynthesis of Flavonoids.

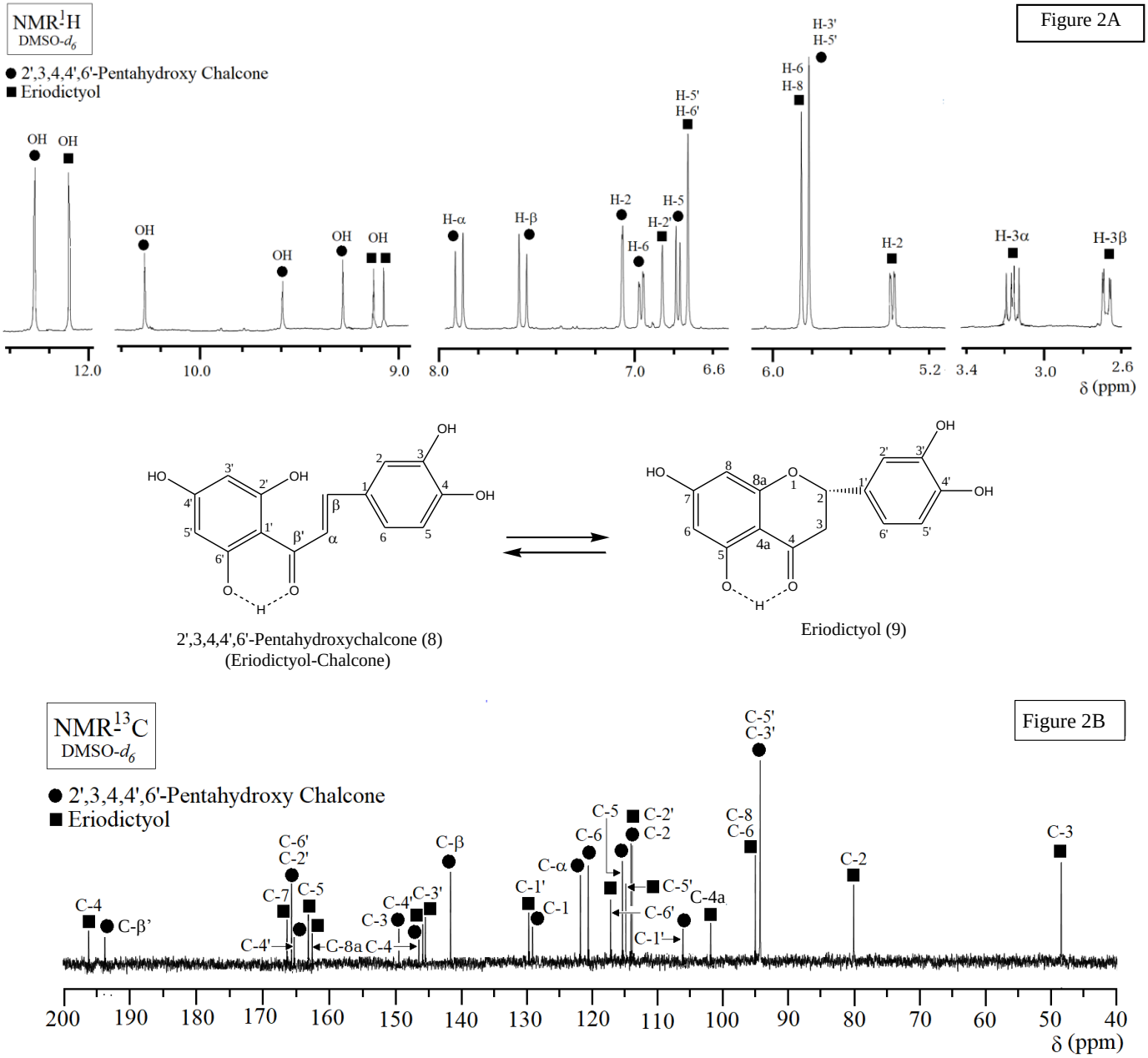

Fig. 2: ^{1}H NMR and ^{13}C NMR spectra of 2quilibrium 2',3,4,4',6' Pentahydroxychalcone (Eriodictyol-Chalcone) (**8**) and Eriodictyol (**9**).

tion was filtered and concentrated *"in vacuo"* in a rotary evaporator at temperature not exceeding 40 ºC, to produce a crude extract (970 g), which was preadsorbed on silica gel and extracted successively with petroleum ether, acetone, and methanol, exhaustively in each case. Acetone-solution was concentrated under reduced pressure to dryness and a brown residue was obtained (≅ 270 g). The acetone extract was preadsorbed on silica gel and chromatographed (VLC) over silica gel 60, eluting with hexane and EtOAc in mixtures of increasing polarity. Fractions of 500 mL were collected and combined according to the TLC characteristics to afford twelve major fractions (A-L). Combined fraction "H" [53-57], eluted with hexane-EtOAc (2:3), was purified by repeated flash chromatography, Sephadex fitration chromatography, preparative TLC and crystallization to furnish an isomorphic crystalline equilibrium mixture of **8** and **9**.

*Equilibrium mixture of **8** and **9***

Liquid recovered by filtration after washing with methanol the scraped silica gel from the preparative TLC of combined fraction "H", was concentrated to dryness yielding a pale yellow solid, whose chromatographic behavior was typical of a flavonoid. Crystallization in methanol gave yellow flakes; m.p. = 262-264 °C. $[\alpha]_D$ = -2.8º (c, 0,5 MeoH). UV, $\lambda_{max.}$: (CH_3OH, nm) 225, 287, 330 (sh); IR, $\nu_{max.}$ (cm^{-1}): 3363 (OH), 1636 (C=O), 1604 (C=C), 1521, 1447, 1160 and 1085 (C-O). EI-MS, *m/z* (%): 288 [M^+] (69), 270 [M^+-H_2O] (14), 179 (24), 166 (40), 163 (18), 153 (100), 152 (12), 136 (70), 123 (16).

^{1}H NMR (400 MHz, DMSO-d_6) Data corresponding to 2',3,4,4',6'-pentahydroxychalcone (**8**)$^{\bullet}$ δ_H: 7.94 (1H, d, *J* = 15.5 Hz, H-α), 7.61 (1H, d, *J* = 15.5 Hz, H-β), 5.88 (2H, s, H-3' and H-5'), 7.11 (1H, d, *J* = 1.7 Hz, H-2), 6.83 (1H, d, *J* = 8.1 Hz, H-5), 7.02 (1H, dd, J = 8.1 and 1.7 Hz, H-6), 12.60 (1H, s, O<u>H</u>), 10.45 (1H, s, O<u>H</u>), 9.66 (1H, s, O<u>H</u>), 9.31 (1H, s, O<u>H</u>); Data corresponding to eriodictyol (**9**)$^{\blacksquare}$ δ_H: 5.41 (1H, dd, *J* = 12.4 and 3.0 Hz, H-2), 3.22 (1H, dd, *J* = 17.1 and 12.4 Hz, H-3αax), 2.71 (1H, dd, *J* = 17.1 and 3.0 Hz, H-3βec), 5.91 (2H, s, H-6 and H-8), 6.91 (1H, bs, H-2'), 6.78 (2H, bs, H-5' and H-6'), 9.08 (1H, s, O<u>H</u>), 9.08 (1H, s, O<u>H</u>), 12.18 (1H, s, O<u>H</u>). ^{13}C NMR (100 MHz, DMSO-d_6) $\delta_C$$^{\bullet}$: 192.5 (>C=O, C-β'), 124.5 (=CH-, C-α), 143.7 (=CH-, C-β), 105.1 (>C=, C-1'), 165.3 (=C-O-, C-2'), 95.8 (=CH-, C-3'), 165.6 (=C-O-, C-4'), 95.8 (=CH-, C-5'), 165.3 (=C-O-, C-6'), 127.6 (>C=, C-1), 115.2 (=CH-, C-2), 146.6 (=C-O-, C-3), 149.4 (=C-O-, C-4), 116.8 (=CH-, C-5), 122.9 (=CH-, C-6); $\delta_C$$^{\blacksquare}$: 79.3 (>CH-, C-2), 43.0 (>CH_2, C-3), 197.2 (>C=O, C-4), 164.4 (=C-O-, C-5), 96.7 (=CH-, C-6), 167.5 (=C-O-, C-7), 96.7 (=CH-, C-8), 163.8 (=C-O-, C-8a), 102.7 (>C=, C-4a), 130.4 (>C=, C-1'), 115.3 (=CH-, C-2'), 146.1 (=C-O-, C-3'), 146.6 (=C-O-, C-4'), 116.3 (=CH-, C-5'), 118.8 (=CH-, C-6').

Results and Discussion

The equilibrium isomeric flavonoid mixture crystallized as yellow flakes; m.p. = 262-264 °C. The EI-MS shows a peak corresponding to a molecular ion at *m/z*: 288 [M^+] which, according to the NMR data, can be assigned to the molecular formula $C_{15}H_{12}O_6$. The presence in its UV spectrum of three bands at λ_{max}: 225, 287, 330 (sh) indicates that it is a chalcone-flavanone mixture[42]. An overview of the ^{1}H NMR and ^{13}C NMR spectra (Fig. 2), clearly shows that it is a mixture of two compounds whose ratio is 5:3 (**8**/**9**). Analyzing both spectra in detail, together with the ^{1}H-^{1}H COSY, HMQC, and HMBC (Fig. 3) 2D-NMR spectra, the chalcone-related signals (identified with the sign "●") and those of the flavanone (identified with the sign "■") could be unequivocally assigned (see experimental; Fig. 2). Next, we will comment and discuss individually the data of each of the compounds that make up the mixture.

Compound (**8**): The molecular formula $C_{15}H_{12}O_6$ revealed ten unsaturation degrees. ^{13}C NMR data analysis (Fig. 2B) allow the characterization in the molecule of functional groups completing eight degrees of unsaturation, a ketone [δ_C 192.5 (>C=O, C-1")] and fourteen sp^2 carbons that form seven double bonds and therefore, the remaining unsaturation should correspond to two rings, forming a system composed of two benzene nuclei. The remaining spectral data allowed to conclude that this compound is the 2',3,4,4',6'-pentahydroxychalcone (eriodictyol chalcone).

This chalcone is not very abundant in Nature, having been sporadically isolated only from four species: *Galium verum* (Rubiaceae)[43], *Limonium cv. 'Gold Coast'* (Plumbaginaceae)[44], *Tulipa cff. "Apeldoorn"* (Liliaceae)[45] and *Sorghum bicolor* (Poaceae)[46], however, it has been detected by GC/MS in the peel of tomato fruits *Lycopersicum sculentum* (Solanaceae)[47], and in some medicinal plants: *Artemisia argyi* (Asteraceae)[48], *Populus sieboldii* (Salicaceae)[49] and *Pouteria lucuma* (Sapotaceae)[50]. An interesting aspect to highlight is the frequency with which its biosynthesis from caffeoyl-CoA (**6**) has been induced in some plants by genetic engineering, using the enzyme chalcone synthase (CHS); in general, plants of nutritional interest have been used: Tomato (*Lycopersicum sculentum*)[51], barley (*Hordeum vulgare*)[52], rice (*Oryza sativa*)[53], carrot (*Daucus carota*)[54], apple (*Malus x domestica*)[55] and pear (*Pyrus communis*)[55], which support the possible interest of this chalcone as a phytochemical additive in the diet to be ingested in human consumption[56].

The spectrum of biological activity of this chalcone has been studied, highlighting its antioxidant activity (Rancimat Test // induction periods in lard at concentrations of 0.025, 0.05 and 0.1% = 20, 40, and 73 h, respectively)[57] and its relative DPPH radical scavenging equivalence (DPPH assay in acetone = 3.8 at 30 min. when tocopherol in ethanol was used as standard)[58]. In an "*in vitro*" study on antimalarial activity, antiplasmodial effects were evaluated observing that (**8**), when it is given at 1 mM concentration, caused a relevant growth inhibition (88%) of 3D7 *Plasmodium falciparum* strain, after 48 hours incubation; in another experiment, the growth of the parasite was investigated when artemisinin, in sublethal doses ranging from 0.625 to 5.0 nM, was added to a 1.0 mM sample of (**8**), detecting considerable additive effects that induce a substantial potentiation of the antimalarial action of artimisinin[59].

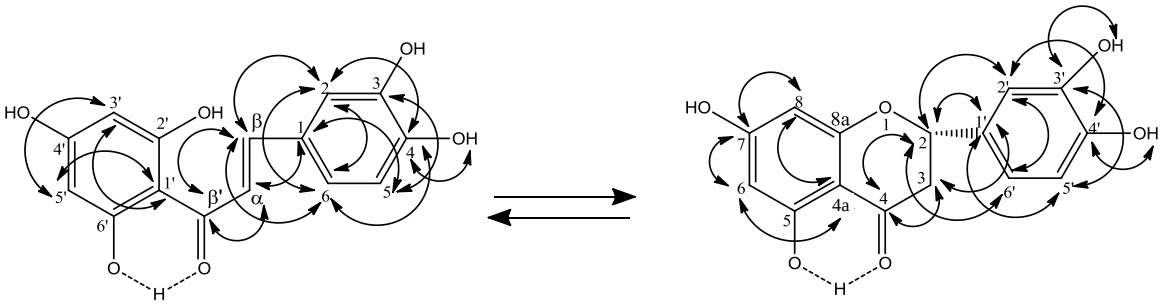

Fig. 3: HMBC Spectrum Correlations of 2',3,4,4',6' Pentahydroxychalcone (Eriodictyol-Chalcone) (**8**) and Eriodictyol (**9**).

Another activity of notable interest is the capacity of (**8**) as an antidepressant agent, which was evaluated "*in vivo*" in mice; the activity of the compound, at a dose of 10 mg/kg, was measured using the forced swimming test, in which it was detected that the mean and standard error of the mean (± SEM) for the duration of immobility of ten mice was 98.5 ± 11.0 sec; this value qualifies (**8**) as a potent antidepressant since it has a $P < 0.01$ against Fluoxetine, a reference antidepressant drug[60]. Finally, it is particularly notable that this chalcone exhibits an inhibitory effect on several enzymes such as phosphoenolpyruvate carboxylase (PEPC) (IC_{50} = 2.5 and 8.2 μM)[61], protein tyrosine phosphatase 1B (PTP1B) (IC_{50} = 1.26 μM)[62], aromatase (CYP19A1) (IC_{50} = 2.8 μM)[63], 5-lipoxygenase (5-LOX) (IC_{50} = 0.043 μM)[64] and cyclooxygenase (COX) (IC_{50} = 34.0 μM)[64]. Consequently, it could be stated that chalcone (**8**) seems to be a potential herbicide for its ability to inhibit PEPC, a promising candidate for the treatment of a variety of inflammatory and allergic diseases due to its action on 5-LOX and COX or a therapeutic approach to prevent the development and progression of breast cancer based on its activity on CYP19A1.

Compound (**9**): The signals (typified as signals ■) in the ^{1}H NMR and ^{13}C NMR spectra corresponding to the second component of the mixture are congruent with those of a flavanone, which was identified with eriodictyol (3',4',5,7-tetrahydroxyflavanone). This flavanone was isolated for the first time in 1906 from *Eriodictyon californicum* (Hooker and Arnott) Greene, a California medicinal plant commonly known as "Yerba Santa"[65], but its structure was not definitively established until 1929, which was also unequivocally supported by its synthesis[66, 67]. Since then, this flavanone has been reported in many higher plants[68] and particularly in species of the genus *Citrus* (Rutaceae)[69].

From the biological and pharmacological point of view, eriodyctiol has a wide spectrum of activities, as can be contrasted when analyzing the recent review of Islam *et al.*[68]; however, it is appropriate to highlight the most recent papers on its anti-oxidants[70], anti-inflammatory[71], cardiovascular[72], and anti-carcinogenic[73,74] activities, as well as the critical role it plays in diabetes mellitus[75]. At this time, it is particularly interesting to note that eriodyctiol is described as an interesting molecule for treating COVID-19, as it is revealed by rigorous studies using a molecular docking approach, via the world's most powerful supercomputer SUMMIT[76]. In these studies, it was confirmed that eriodictyol might affect the life cycle of the SARS-CoV-2 virus by interacting with various amino acids and proteins, binding to multiple targets with efficient binding energy (between -6.7 and -8.8 kcal/mol) and within the active site[77]; consequently, eriodictyol can become a multi-target molecule which can be a strong candidate for the treatment of coronavirus infection when repurposed against SARS-CoV-2.

In accordance with all the above and in harmony with the title of this article, it is appropriate to make some comments about the chalcone-flavanone equilibrium. The scientific literature reveals that it is rare to find this equilibrium type in the same plant species; however, the presence of the equilibrium 2',3,4,4',6'-pentahydroxychalcone (eridictyol chalcone)-eriodictyol [(**8**) ⇆ (**9**)], has been reported in certain common plants such as tomato (*Lycopersicum sculentum*, Solanaceae)[47], sorghum (*Sorgum bicolour*, Poaceae)[46] and poplar *Populus sieboldii*, Salicaceae)[49] and also in incubations with plant enzyme preparations obtained from fresh vegetable materials such as flowers of defined genotypes of carnation (*Dianthus caryophyllus*, Caryophyllaceae)[78] or anthers and pollen of tulips (*Tulipa cff. "Apeldoorn"*, Liliaceae)[79].

The synthetic cyclization of chalcones into flavanones and vice versa has been widely studied[80-82]. Both processes have been carried out chemically using acids [acetic acid, trifluoroacetic acid, methane sulphonic acid, amino acids, Lewis acids and mineral acids such as HCl, H_2SO_4, H_3PO_4, polyphosphoric acid, etc.], bases [alkali metal hydroxydes, sodium acetate, potassium carbonate in acetone under reflux condition, amine bases, cobalt(II) Schiff-base], aqueous buffers at variable pH, acidic ion-exchange resin, phase-transfer catalysts, catalysis by Co(III)-salts complexes, bovine serum albumin (BSA), oxidizing agent as potassium ferricyanide, silica gel, zeolites, celite supported potassium fluoride, Amberlyst A-21, photochemical processes, thermal reactions at 60 °C in the solid-state or in the melt at 230 °C, microwave irradiation or under electrochemical conditions. It should be noted that the chalcone-flavanone equilibrium conforms a scaffold that can function as a dynamic covalent molecular switch, that is, a molecule that is capable of being reversibly biased towards two states in response to an external stimulus[83], the development of these systems (the so-called "molecular switches") have been a mature area of research during the last decade, as they have application in a variety of fields including sensors, chemical biology, and functional materials[84]. Finally, the cyclization of chalcones can lead to flavonoids other than flavanones; an example of this is the transformation of the eriodictyol chalcone (**8**) into the aurone aureusidin, through a crude peroxidase, polyphenol oxidase (PPO)[85].

As conclusion, it is undeniable to say that in our case since we never use acids or bases in the extraction and isolation processes, the coexistence of the chalcone-flavanone equilibrium is natural, and must be regulated by the action of the enzyme chalcone isomerase (CHI). This equilibrium, being photosynthetically originated, could be considered a dynamic covalent photoswitch.

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Conflicts of Interest: The authors have no conflicts of interest to declare.

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