ABSTRACT

RONGHUI, WANG. Amination, a Newly Discovered Metabolic Pathway of Myricetin and Baicalein. (Under the direction of Dr. Shengmin Sang and Dr. Jonathan C. Allen).

Many studies have shown that dietary flavonoids, which are abundant in human diets, have significant antioxidative properties and may prevent cancer and cardiovascular diseases. However, there is still a wide gap in the understanding of flavonoid activities and their practical application due to the low bioavailability of most dietary flavonoids and our deficient knowledge of the *in vivo* biotransformation mechanism of these dietary flavonoids. Previous studies found that polyphenols with a *vic*-trihydroxyl group, such as epigallocatechin gallate (EGCG), epigallocatechin (EGC), and pyrogallol, have the capacity to chemically react with ammonia and generate aminated products. Ammonia is primarily a waste product of cellular metabolism *in vivo* and maintains at concentrations less than 50 μM in healthy adults. The overall goal of this project is to determine whether amination is a new metabolic pathway of flavonoids with a *vic*-trihydroxyl group using myricetin and baicalein as examples. In this study, we demonstrated for the first time that myricetin, a flavonol with a *vic*-trihydroxyl group on the B ring, could chemically react with ammonia to generate the aminated product *in vitro* and in mice. As we expected, the amination occurred on the position 4 of the B-ring of myricetin. The structure of this new metabolite was confirmed based on 1D and 2D NMR and LC-MS spectral analysis. Furthermore, we found that baicalein, a flavone with a *vic*-trihydroxyl group on the A ring, could also react with

ammonia to form two mono-aminated and one di-aminated products. The two mono-aminated products were identified as the major metabolites of baicalein in mice. This is the first study to confirm that amination is a newly discovered metabolic pathway for myricetin and baicalein. It is worthwhile to study whether amination is the metabolic pathway for other flavonoids or polyphenols with a *vic*-trihydroxyl group and to further study the bioactivities of the aminated metabolites. This novel metabolic pathway will advance our knowledge on the biotransformation of dietary flavonoids.

Amination, a Newly Discovered Metabolic Pathway of Myricetin and Baicalein

by
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DEDICATION

To those who support me and love me in my life.

BIOGRAPHY

Ronghui Wang is from a warm, sweet, small family in the capital of eastern China's Shandong province. As the only child, Ronghui was surrounded by love from all of his family members. When he was eight, his uncle, a neurosurgeon, gave him a binocular biological microscope as a birthday gift. After that, Ronghui fell in love with the world of science. He read every issue of We Love Science and found insects, leaves, or even tadpole eggs to be tremendously exciting under the microscope.

After graduating from Shandong Experimental High School, Ronghui began his bachelor's degree in Food Science and Engineering at Shandong Agricultural University. He was a part of many school activities, became a volunteer teacher in west China, worked as an outstanding volunteer for the Red Cross, and joined his college debate team. In his senior year, Ronghui participated in two research projects: Optimization of Modified Atmosphere Packaging to Influence the Microorganisms and Quality of Chilled Beef and Preparation and Characterization of Starch-HPMC Nanocomposite Films.

After graduating Ronghui began his master's degree in Food Science at NC State University under the direction of Dr. Shengmin Sang. After the first year of courses, he conducted research on the metabolism of bioactive food components in Dr. Sang's lab at North Carolina Research Campus, Kannapolis.

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CHAPTER 1: Introduction

1.1 Dietary flavonoids and their health effects

Flavonoids, the most common group of polyphenolic compounds, widely occur in foods of plant origin. Over 4000 different kinds of flavonoids have been discovered and described in previous studies. There are six major subclasses of flavonoids divided by the variations in the heterocyclic ring C, such as flavonols, flavones, flavanols, flavanones, anthocyanidins, and isoflavones. From the recent data analyses of the National Health and Nutrition Examination Survey (NHANES), the estimated mean flavonoid intake in US adults (aged ≥19 years) was 344.83 ± 9.13 mg/day [1]. The top five major dietary flavonoid sources are tea, citrus fruits and juices, wine, berries, and apples. For example, tea contributes approximately 157 mg of daily flavonoid intake [2], in other words, there are 52- 133 mg flavonoids per 100 mL brewed tea [3].

Over the past 10 years, researchers and manufacturers have had an increased amount of interest in flavonoids because of their diverse biological activities, such as antioxidative, anti-inflammatory, anti-allergic, anti-carcinogenic, and cardiovascular protection, which have been demonstrated in previous studies [4-6]. Many of the flavonoids, such as tea catechins and quercetin, have been reported to have anti-inflammatory activity by inhibiting cycloxygenase-2 and inducible nitric oxide synthase [7]. From a human study reported by Bondonno, et al., apple flavonoids were found to be able to significantly improve endothelial functions which has been linked to inhibition of atherosclerosis, stroke, and other

cardiovascular diseases [8]. Shabrova, et al., found that cranberry supplementation, containing myricetin, was able to improve insulin sensitivity and increase adiponectin levels, known for its anti-diabetic and anti-atherogenesis effects, in obese mice [9]. There are many other articles that have been published describing the anti-carcinogenic properties that flavonoids possess [10-13].

1.2 Myricetin and its health effects

Myricetin is a member of the flavonol subclass, with hydroxyl substitutions at the 3, 5, 7, 3´, 4 ´, and 5´ positions. Myricetin was originally isolated from the bark of Myrica nagi in the late eighteenth century [14]. This compound has been reported from a wide range of plants including many different foods, such as berries, vegetables (spinach: 1660.9 mg/kg, carrot: 523.3 mg/kg, peas: 146.2 mg/kg), fruits, nuts, tea (black tea: 0.2-0.5 g/kg, green tea: 0.8-1.6 g/kg) [15], red wine, and medicinal herbs [16]. It has been reported that the average intake of five major dietary flavonoids (quercetin, myricetin, kaempferol, luteolin, and apigenin) was 23 mg/day in Netherlands [17].

Recent pharmacological studies have shown that myricetin possesses bioactive properties, such as having antioxidative, anti-carcinogenic, and anti-proliferative effects [18]. Researchers found that myricetin causes cytotoxicity towards a variety of human cancer cells lines, such as colon, hepatic, and skin; moreover, it was found to also inhibit key enzymes related to carcinogenesis [19]. Myricetin, at a 3 mg/kg/12 hr dose, was also found to

significantly reduce hyperglycemia and increase hepatic glycogen and glucose- 6-phosphate in streptozotocin-induced diabetic rats; additionally, myricetin may be able to reduce insulin sensitivity by enhancing the PI3-kinase and GLUT 4 activity [20]. Moreover, myricetin was reported to ameliorate insulin resistance induced by high-fructose diet in rats. The rats were given myricetin 3 times/day (1 mg/kg body weight, i.v.) for 2 weeks. The defect in expression of insulin receptor substrate-1 (IRS-1) and the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) in the soleus muscle of fructose chow-fed rats was reversed by the myricetin treatment [21].

1.3 Baicalein and its health effects

Baicalein (5, 6, 7-trihyroxyflavone) is a flavone present in the roots of *Scutellaria baicalensis* Georgi (also as known as the Chinese Huang Qin) [22]. *Scutellaria baicalensis* Georgi is one of the most common and multi-purpose herbs used in Traditional Chinese Medicine and dietary supplements. Baicalein comprises 5.41% of the raw material weight gathered from the plant [23]. The record for this plant can be found as an important ingredient of Xiao Chai Hu Tang recipe dated back to 200 AD, described in Shang Han Lun (Treatise on Cold Damage Disorders), which was written by Zhong Jing Zhang, an eminent Chinese physician. This supplement recipe was described as a treatment for colds, fevers, and depression. The *vic*-trihydroxyl functional group of baicalein is vital for its pharmacological effects, which resulted in manufacturers and researchers becoming increasingly interested in this compound due to its potential health benefits [24]. So far,

baicalein has been intensively studied *in vitro* and *in vivo* due to its strong anticancer effects. Baicalein has demonstrated potent apoptosis-inducing properties in various types of cancer cells. One of the main mechanisms to regulate cellular proliferation is the apoptotic pathway due to baicalein generating reactive oxygen species to induce apoptosis [25]. In an *in vivo* experiment, different doses of baicalein (control, 0.05 mg, 0.1 mg) were given to C3H/HeN mice implanted with Murine bladder cancer cell line; the tumor volume was observed from day 1 to day 30 with size measurements on day 20 and 30. The tumor size of the control group on day 20 and 30 was 2.23 ± 0.32 cm^3 and 5.03 ± 1.22 cm^3, respectively. After the administration of 0.05 mg of baicalein, the tumor size on day 20 and 30 was 1.6 ± 0.44 cm^3 and 3.7 ± 1.55 cm^3, respectively. Compared to the control group, the baicalein treated group significantly inhibited tumor growth within the mice, and the results were even more prominent for the group administered 0.1 mg of baicalein [26].

1.4 Bioavailability and biotransformation of dietary flavonoids

In general, bioavailability is a term to describe the degree or rate at which a drug/food is absorbed by humans' circulatory system. Bioavailability is a major factor used to assess the biological properties of a dietary compound. The health effects of flavonoids depend on the amount consumed and their bioavailability. However, the bioavailability of most flavonoids is poor in most cases. For instance, a typical cup of green tea contains around 88 mg epigallocatechin gallate (EGCG), but only 0.33 μM of EGCG could be detected in plasma

after ingesting a total quantity of it, demonstrating the low bioavailability of dietary flavonoids *in vivo* [27]. One of the main reasons for the poor systematic bioavailability of flavonoids is that they are extensively metabolized *in vivo*. Biotransformation is the process of metabolizing the parent compound into its metabolite form. Flavonoids are known to be metabolized by phase I and phase II enzymes, and the gut microbiota.

Phase I reaction is the chemical modification by cytochrome P450 enzymes, which performs oxidative, reductive, or hydrolytic reactions to generate hydroxyl groups, epoxides, thiols, and amines. For example, in a rat liver microsomes study, galangin, a type of flavonoid, was found to be metabolized to the 4´-hydroxylated product, which means one hydroxyl group was added at the C 4´position [28].

Phase II reactions mainly comprise methylation, glucuronidation, sulfation and GSH conjugation, which are the main metabolic pathways for most of the dietary flavonoids. For example, in a metabolite identification study of myricetin in rats, myricetin was administrated to rats by oral gavage, and plasma, urine and feces samples were collected, based on the analysis of LC-MS spectra, seven metabolites were identified and five of them (M3-M7) were the Phase II metabolites of myricetin. M3, M4, and M5 were the mono-, di- and tetra-methylated myricetin, respectively. M6 and M7 were the mono- and di-glucuronidated products of myricetin [29].

Gut microbiota metabolism is primarily catabolism. The catalytic and hydrolytic potential of bacteria towards dietary components is exceptional due to the 1000 different bacterial species which comprise 100 trillion bacteria. Gut microbiota catalyzed the

breakdown of flavonoids into simple compounds [30]. For example, it has been reported that EGCG, the most abundant compound in green tea, could be metabolized by gut microbiota to generate the ring fission products 5-(3′,4′,5′- trihydroxyphenyl)-γ-valerolactone, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, and 5- (3′,5′dihydroxyphenyl)-γ-valerolactone [31].

Dietary flavonoids exist mainly in their metabolite forms. Increasing evidence has shown that flavonoids are highly bioavailable if we consider the total levels of flavonoids and their metabolites [32]. However, the metabolic profiles of most of the flavonoids are not fully understood. In addition, the bioactivities of these metabolites are largely underinvestigated. Therefore, there is a need to further study the metabolisms of flavonoids with the hope to better understand their beneficial health effects.

1.5 Animation of polyphenols

Tea catechins, a type of disease-fighting flavanol, has been reported to have deodorizing effects on amines and ammonia [33]. From a previous study, epigallocatechin (EGC) was used to react with amines and ammonia, respectively, and the reaction products were identified as the aminated EGC with the hydroxyl group at 4′ position of EGC replaced by an amino group [34]. Furthermore, a study in Japan showed that compounds containing a pyrogallol nucleus (a *vic*-trihydroxyl group) such as pyrogallol and gallic acid could also be able to react with ammonia to form the aminated products [35]. However, flavonoids that contain a catechol nucleus (a *vic*-dihydroxyl group) are unable to undergo amination via

ammonia treatment [35].

Ammonia is ubiquitous within our bodies and is a waste product of cellular metabolism. Most ammonia is generated from the intestines, from the digestion of protein and lysis of urea [36]. Normally, *in vivo* ammonia is maintained at a balanced level by certain organs such as liver, at concentrations ＜50 μM in adults [37]. However, high concentrations of ammonia accumulate when the hepatic detoxification process becomes impaired. Recently, clinical treatments of hyperammonemia are mainly divided into two parts: reducing ammonia generation and uptake and increasing ammonia removal ability. For the second part, several therapeutic agents were used to support *in vivo* ammonia detoxifying pathway, such as L-ornithine, L-aspartate, and carglumic acid [38]. It is, therefore, worthwhile to determine whether flavonoids with the pyrogallol nucleus (a *vic*-trihydroxy group) can react with endogenous ammonia to generate aminated metabolites. This determination will lead to a better understanding of degradation pathways by which ammonia can be expelled from the body and aid in the treatment of hyperammonemia induced conditions. Also, the identification of possible metabolites formed from this interaction could be examined for possible health-promoting potentials.

CHAPTER 2: HYPOTHESES AND OBJECTIVES

2.1 Hypothesis

Previous studies found that polyphenols with a *vic*-trihydroxyl group, such as EGCG, EGC, and pyrogallol have the capacity to chemically react with ammonia and generate aminated products. Ammonia is primarily a waste product of cellular metabolism *in vivo* and is maintained at a concentration <50 μM in healthy adults [39]. Therefore, our hypothesis is that amination is a metabolic pathway of polyphenols with a *vic*-trihydroxyl group *in vivo*. To test this hypothesis, we studied the formation of aminated products of two important flavonoids, myricetin, which has a *vic*-trihydroxyl group on B-ring, and baicalein, which has a *vic*-trihydroxyl group on A-ring, *in vitro* and *in vivo*.

2.2 Research Objectives

The overall goal of this project is to determine whether amination is a "new" metabolic pathway for flavonoids with a *vic*-trihydroxyl group using myricetin and baicalein as examples. To achieve this goal, we have the following two aims:

1. To chemically synthesize aminated myricetin and baicalein products *in vitro* and characterize their structures by spectroscopic analysis.
2. To determine whether the myricetin and baicalein are aminated in mice.

CHAPTER 3: Animation as a Newly Discovered Metabolic Pathway of Myricetin

3.1 Materials & Methods

3.1.1 Materials

Myricetin was purchased from AK Scientific Inc., Union City, California. Ammonium hydroxide solution and hydrochloric acid were purchased from Sigma-Aldrich, St. Louis, Missouri. Methanol (ACS) was purchased from VWR, Radnor, Pennsylvania. Methanol (HPLC) was purchased from Fisher Scientific, Hampton, New Hampshire. All deionized water used was produced by the NANOpure® Dlamond™ Life Science (UV/UF), purchased from Thermo Fisher Scientific Inc., Waltham, Massachusetts.

3.1.2 Time course

Based on what was reported in the literature, polyphenols with a *vic*-trihydroxyl group could chemically react with ammonia to generate the aminated products [35]. In that study, the authors used a very high concentration of ammonia (10 M) and a reaction time of 60 min. Since flavonoids are not stable in a basic medium, the reaction conditions were checked with a lower concentration of ammonia and a shorter reaction time to achieve the desired results. To identify the best reaction time for the aminated myricetin products, 10 mg of myricetin was added into 4 mL of 5 M ammonium hydroxide and stirred at room temperature. Then 200 µL of reaction solution was taken out at 0, 5, 10, 20, 30, and 60 min, respectively. After

incubation, 6 M hydrochloric acid was used to stop the reaction (final pH 5~6). Then the reaction solution was diluted two times with methanol. After centrifugation, the supernatant was taken out for HPLC-DAD analysis to check the formation of the products. The peak areas of the products were compared to determine the best reaction time.

3.1.3 Large scale of reaction

Using the optimized reaction condition, 100 mg of myricetin was added into 40 mL of 5 M ammonium hydroxide and stirred for 20 min at room temperature. 6 M hydrochloric acid was used to stop the reaction (final pH 5~6). Then, 50 µL of reaction solution was diluted two times with methanol. After centrifugation, the supernatant was taken out for HPLC-DAD to confirm the formation of the products. The reaction solution was dried by rotary evaporator and reconstituted in 100% methanol. The purification of the final aminated product was performed with preparative-HPLC.

3.1.4 HPLC-DAD analysis

HPLC analysis was carried out with a Thermo Scientific Dionex Ultimate 3000 system which consisted of an Ultimate 3000 RS pump, an Ultimate 3000 RS autosampler, and an Ultimate 3000 Diode Array Detector (Thermo Fisher Scientific Inc., Waltham, Massachusetts). A Gemini 5 µm C18 110A column (150 mm × 4.6 mm, 5µm; Phenomenex, Torrance, California) was used to detect the products of reaction. The mobile phases

consisted of solvent A (95% deionized water and 5% methanol containing 0.1% formic acid) and solvent B (95% methanol and 5% water containing 0.1% formic acid). The gradient elution had the following profile: 5% B from 0 to 5 min; 5-100% B from 5 to 35 min; 100% B from 35 to 37 min; 100-0% B from 37 to 40 min; and 0% B from 40 to 45 min. The flow rate was 1 mL/min. The eluent was detected by DAD with wavelength settings at 210, 275, 375, and 460 nm.

3.1.5 Preparative HPLC

HPLC preparation was carried out with a Waters preparative HPLC system which consisted of a Waters 2489 UV/Visible Detector (Waters Inc., Milford, Massachusetts) and a Waters 2535 Quaternary Gradient Module (Waters Inc., Milford, Massachusetts). A Luna C18 (2) 100A column (250 mm × 10 mm, 5µm; Phenomenex, Torrance, California) was used to separate the products of reaction. The mobile phases consisted of solvent A (95% water and 5% methanol containing 0.1% formic acid) and solvent B (95% methanol and 5% water containing 0.1% formic acid). The gradient elution had the following profile: 45% B from 0 to 5 min; 45-70% B from 5 to 10 min; 70% B from 10 to 13 min; 70-45% B from 13 to 15 min. The flow rate was 3 mL/min. The eluent was detected by UV/Visible Detector with wavelength settings at 210 and 275 nm, and the peak containing aminated product was collected and dried by rotary evaporator. The purity of the aminated product was checked by analytical HPLC.

3.1.6 LC-MS analysis

LC-MS analysis was performed using a Spectra system consisting of an Ultimate 3000 degasser, an Ultimate 3000 RS pump, an Ultimate 3000 RS autosampler, an Ultimate 3000 RS column compartment, and an LTQ Velos Pro ion trap mass spectrometer (Thermo Electron, San Jose, California) equipped with an electrospray ionization (ESI) interface. Chromatographic separation was performed using a 150 mm × 3.0 mm i.d., 5 µm, Gemini C18 column (Phenomenex, Torrance, California). The mobile phases consisted of mobile phase A (95% LC-MS grade water and 5% methanol with 0.1% formic acid) and mobile phase B (95% aqueous methanol with 0.1% formic acid). For the determination of the polyphenols, the gradient elution was performed for 45 min at a flow rate of 0.3 mL/min using the following gradient: 5% B from 0 to 5 min, 5-100% B from 5 to 35 min, 100% B from 35 to 37 min, 100-0% B from 37 to 40 min, and 0% B from 40 to 45 min. The injection volume was 10 µL. The negative ion polarity mode was set for an ESI ion source with the voltage on the ESI interface maintained at approximately 3.6 kV. Nitrogen gas was used as the sheath gas at a flow rate of 34 arbitrary units (AU) and the auxiliary gas at 10 AU. The collision- induced dissociation was conducted with an isolation width of 1.0 Da and a normalized collision energy of 35 for MS/MS analysis. The data were acquired by SIM (Selected-ion monitoring) mode with Xcalibur version 2.0 (Thermo Electron).

3.1.7 NMR analysis

The purified aminated myricetin product was dissolved in 200 µL of d6-DMSO. ^{1}H-(600 MHz), ^{13}C-NMR (150 MHz), and all 2D NMR spectra including HMQC (Heteronuclear Multiple-Quantum Correlation) and HMBC (Heteronuclear Multiple Bond Correlation) were acquired on a Bruker Avance 600 MHz NMR instrument (Bruker Inc., Silberstreifen, Rheinstetten, Germany). Multiplicities were indicated by s (singlet), d (doublet), and m (multiplet). The ^{13}C-NMR spectra were proton decoupled.

3.1.8 Animal study

Male CF-1 mice (5-6 weeks old) were purchased from Charles River Laboratories, Inc., Wilmington, Massachusetts. The mice were acclimated for at least 1 week before the experiment. The experimental animal protocol was approved by the Institutional Animal Care and Use Committee of the North Carolina Research Campus (No.16-016). The mice were housed (5 mice/cage) and kept in air-conditioned quarters with a room temperature of 20 ± 2 °C, relative humidity of 50 ± 10%, and a light: dark cycle of 12:12 h (7 am to 7 pm). The mice were allowed free access to water and were fed a normal mouse diet.

Two groups of mice (4 mice/group) were washed out for three days by feeding the AIN-93G diet. After fasting overnight, one group was administered with myricetin (50 mg/mL in DMSO) by oral gavage at the dose of 200 mg/kg body weight. Another group was treated with the same volume vehicle (DMSO). After oral gavage, two groups of the mice

were moved into the metabolic cage separately to collect the urine and feces for 24 hours. The urine and stool were stored at -80 °C before analysis.

3.1.9 Preparation of the *in vivo* samples

Feces: 100 mg of feces were homogenized in 1 mL of 80% methanol with 0.1% formic acid (FA). The homogenizer used for fecal sample preparation is OMNI Bead Ruptor 24 (Omni International, Kennesaw, Georgia). The samples were homogenized at the speed of 3.55 for 1 min 01 second at 4 °C, C=10, D=0:11. The homogenized suspension was extracted by sonication for 30 minutes. After centrifugation, the extracts were dried by nitrogen. After drying, the extract was re-dissolved in 200 µL of 80% methanol with 0.1% FA and centrifuged for 15 min at 16.1 g, then analyzed by LC-MS.

Urine: 200 µL of urine was mixed with 1 mL acetonitrile, vortex, standing for 10 minutes. After centrifugation, the supernatant was dried by nitrogen, reconstituted in 100 µL of 80% methanol with 0.1% FA and centrifuged for 15 min at 16.1 g, then analyzed by LC-MS.

Hydrolysis of urine: frozen urine samples were taken out of the -80 °C freezer and warmed at room temperature for approximately 1 h. Enzymatic deconjugation of the urine samples was conducted according to our previously published method with slight modification [40]. In brief, 200 µL of urine samples from each group were treated with a mixture of glucuronidase (500 U) and sulfatase (6 U) for 2 hours at 37 °C. SPE was used to

remove the enzymes and proteins or salts in urine from the reaction solution and the metabolites were collected by eluting with methanol. The SPE process was carried out with Oasis® HLB 96-Well Plate 30 µm (30 mg) from Waters Inc., Milford, Massachusetts. The column was washed by 1 mL 100% methanol, then, 1 mL water was used to balance the column. After loading the sample, the column was washed by 1 mL water to remove the untargeted components and 1 mL methanol was used to get the final elute. The elute was dried by compressed nitrogen and re-dissolved in 50 L of 80% methanol and analyzed by LC-MS.

3.2 Results

3.2.1 Optimization of reaction time

Samples were taken at 0, 5, 10, 20, 30, and 60 min of the reaction between 10 mg of myricetin and 4 mL of 5 M ammonium hydroxide at room temperature. As shown in Figure 1, myricetin completely reacted with ammonia within 5 min. Starting from 30 min, the amount of the major product produced decreased. At 60 min, around 80% of the product disappeared, indicating that this product was not stable. Therefore, we chose 20 min as the reaction time for the big scale reaction.

3.2.2 Purification and characterization of the aminated product of myricetin

For the large-scale reaction, 100 mg of myricetin was reacted with 40 mL of 5 M ammonium hydroxide for 20 min at room temperature. The chromatogram of the reaction profile was shown in Figure 2. As expected, one major product was generated. This product was purified by preparative HPLC. At the end of the study, 13 mg of the pure product was obtained. The molecular weight of this aminated product was checked by LC-MS. The result showed its molecular weight under ESI negative mode was m/z 316 $[M-H]^-$, which was one Dalton less than myricetin.

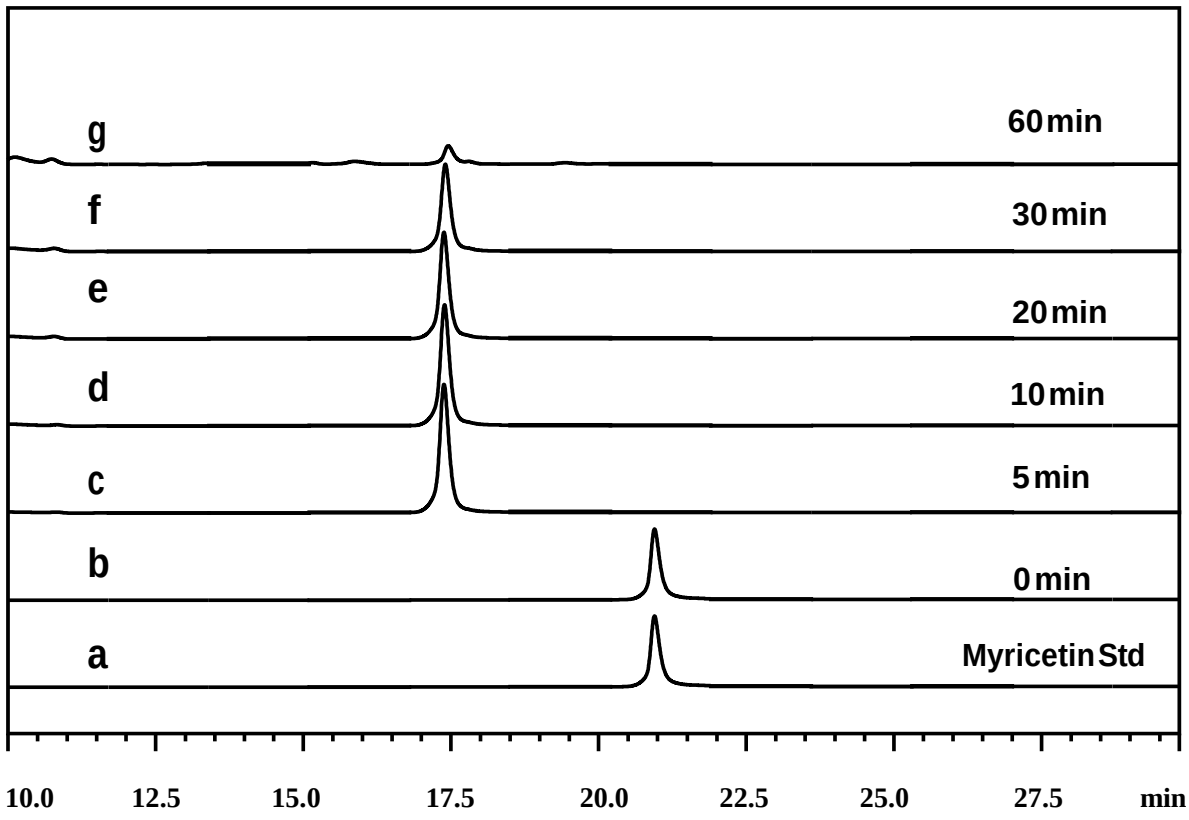

Figure 1. HPLC-DAD chromatograms of the reaction products of myricetin with 5 M of ammonium hydroxide at different reaction times. a) The standard of myricetin. b-g) The

product from reaction for 0 (b), 5 (c), 10 (d), 20 (e), 30 (f), and 60 min (g).

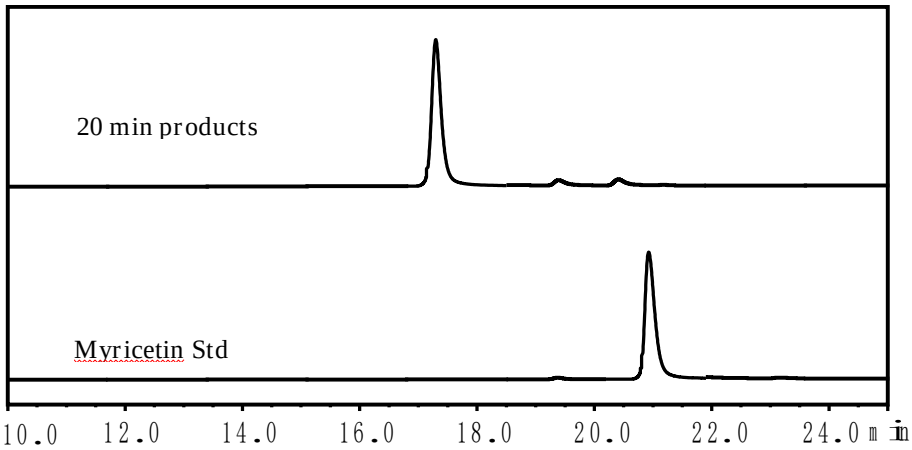

Figure 2. HPLC-DAD chromatograms of the myricetin standard and the large scale reaction of myricetin and 5 M ammonium hydroxide for 20 minutes.

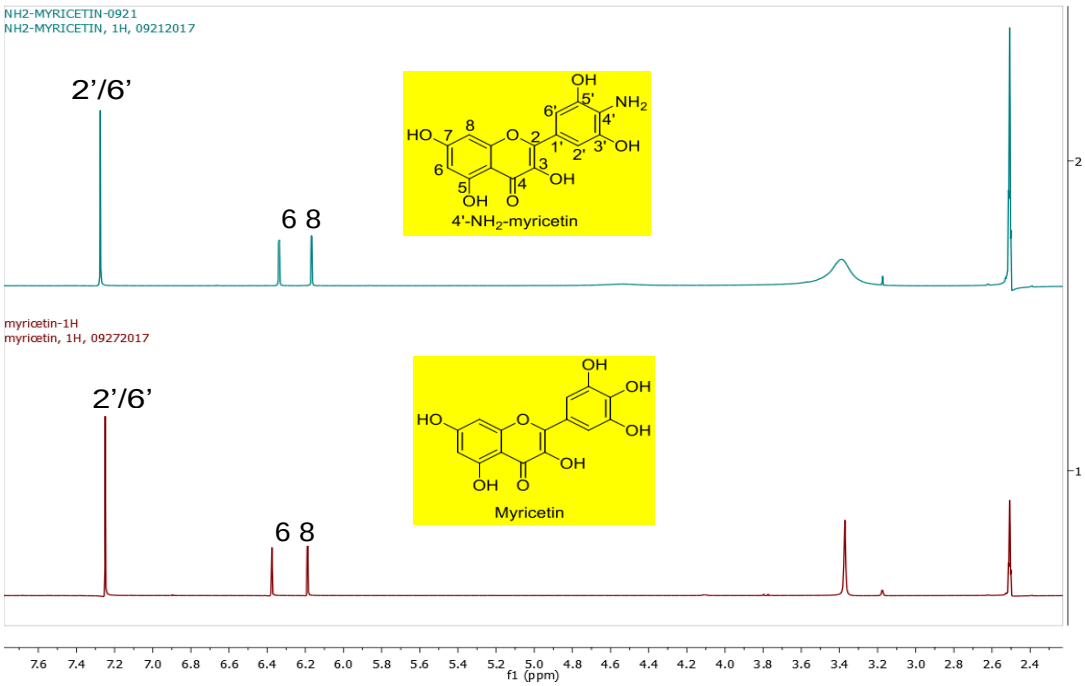

Figure 3. The ^{1}H -NMR spectra of myricetin and its aminated product in d6-DMSO.

In its ^{1}H-NMR spectrum (Figure 3), the protons appeared at δ_H 6.34 (1H, d, 2.0), 6.17 (1H, d, 2.0), and 7.29 (2H, s) showed the same chemical shifts and coupling pattern with those of myricetin indicating there was no change at H-6, H-8, and H-2´6´ of myricetin. However, comparing the carbon NMR spectra of myricetin and its aminated product (Figure

4), the chemical shifts of all B-ring carbons were shifted to upfield, with C-4´ had the largest change from 135.8 ppm to 127.6 ppm. This indicated that on B-ring the high electronegativity substitute, hydroxyl group, was replaced by the low electronegativity substitute, amino group. In order to confirm the exact position of this amino group, its 2D NMR spectra were recorded including HMQC (Heteronuclear Multiple-Quantum Correlation) and HMBC (Heteronuclear Multiple Bond Correlation) experiments. The HMBC correlations from H-2´/6´ at δ_H 7.29 to the carbon at δ_C 127.6 confirmed this upfield shifted carbon was C-4´. Careful analysis of its HMQC and HMBC data allowed the assignment of all proton and carbon signals (Figure S3, S4 and Table 1). Therefore, this aminated product was elucidated as 4´-NH_2-myricetin (Figure 5).

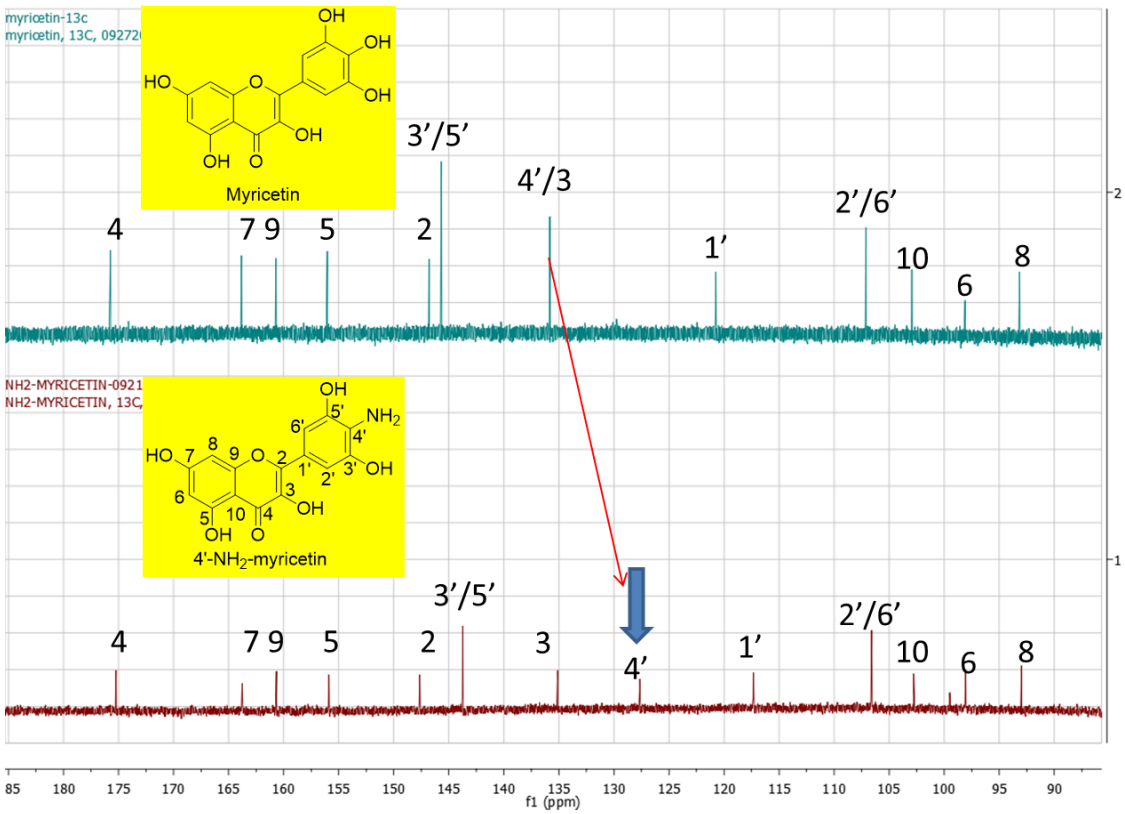

Figure 4. The ^{13}C-NMR spectra of myricetin and its aminated product in d_6-DMSO.

Table 1. ^{1}H-(600 MHz) and ^{13}C-NMR (150 MHz) data of 4´-NH_2-myricetin (in d6-DMSO, δ in ppm, J in Hz)

No.	δ_H	δ_C
2	-	147.6
3	-	135.1
4	-	175.3
5	-	155.9
6	6.34 (d, 2.0)	98.1
7	-	163.8
8	6.17 (d, 2.0)	93.0
9	-	160.7
10	-	102.7
1´	-	117.3
2´	7.29 (s)	106.6
3´	-	143.7
4´	-	127.7
5´	-	143.7
6´	7.29 (s)	106.6

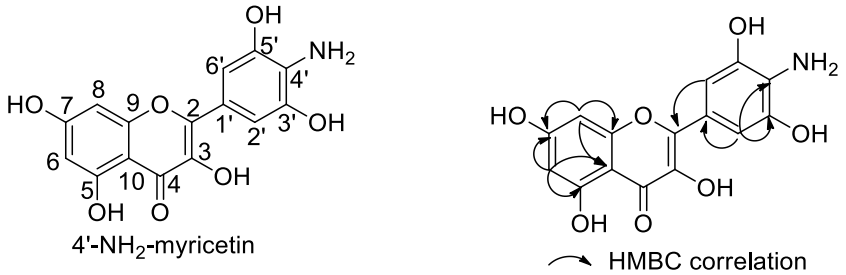

Figure 5. The structure of 4´-NH_2-myricetin and its key HMBC correlations

3.3.3 *In vivo* animal study

The urine and fecal samples collected from myricetin-treated CF1 mice were analyzed by LC-MS/MS and compared with the synthesized standard. The aminated myricetin was not detected in urine samples collected from myricetin-treated mice. But in the mouse fecal samples, one clear peak under molecular weight at m/z 316 $[M-H]^-$ showed the same retention time and tandem mass spectra with those of the synthetic standard, 4´-NH_2-myricetin (Figure 6). This result indicated that under *in vivo* conditions, myricetin could be oxidized to its ortho-quinone and then further react with ammonia through nucleophilic addition to produce the aminated metabolite in the GI tract. The reaction mechanism was shown in Figure 7. In addition, we also proposed the fragmentation pathway of aminated myricetin in Figure 8.

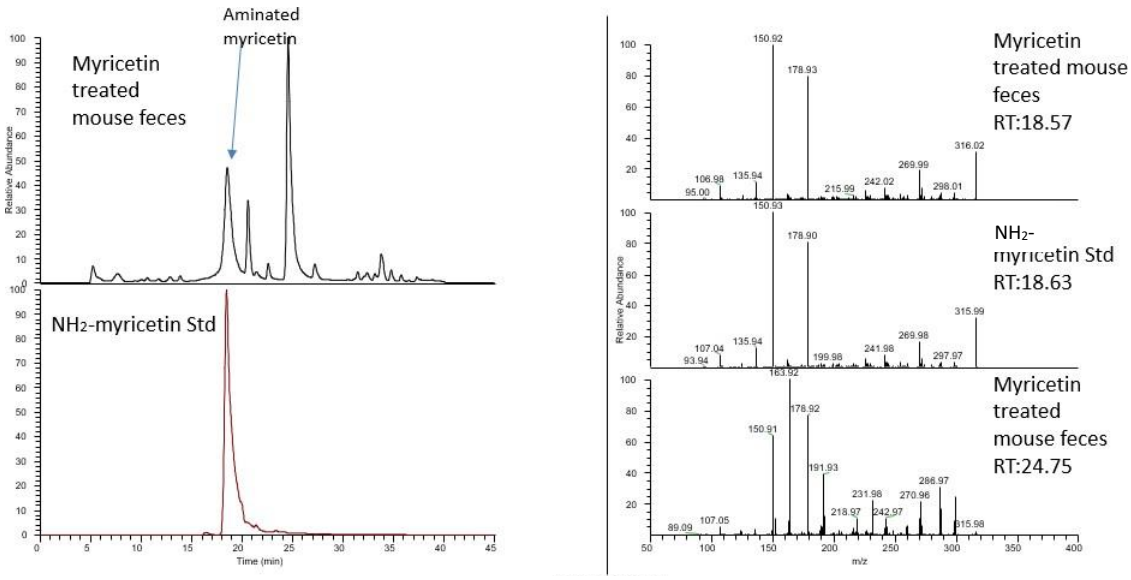

Figure 6. LC chromatograms of 4´-NH_2-myricetin standard and the peaks in mouse fecal samples under selected ion monitoring (SIM) mode and their corresponding MS/MS (negative ion) spectra.

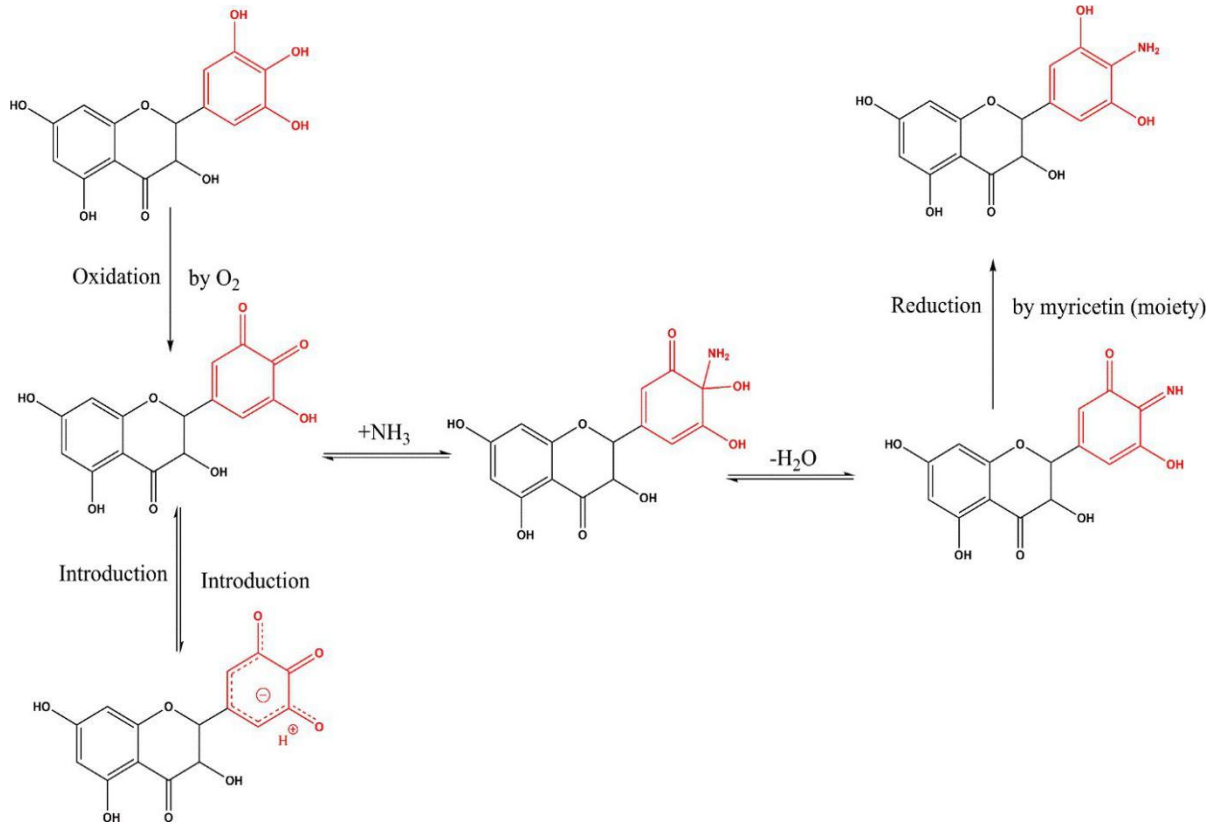

Figure 7. Chemical reaction mechanism of myricetin with ammonia

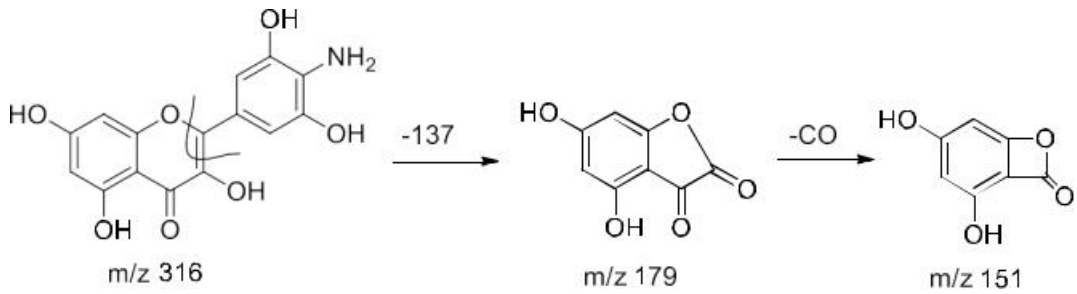

Figure 8. The proposed fragmentation pathway of 4´-NH_2-myricetin

3.3 Conclusion

In summary, we demonstrated for the first time that myricetin could rapidly react with ammonia to produce the aminated myricetin, and this chemical reaction could also occur *in*

vivo. Our LC-MS and NMR analysis clearly showed that the amination occurred on the 4´-carbon of the B-ring. Therefore, our results support our hypothesis that amination is a metabolic pathway of polyphenols with a *vic*-trihydroxyl group *in vivo*.

CHAPTER 4: Amination as a Newly Discovered Metabolic Pathway of Baicalein

4.1 Materials & Methods

4.1.1 Materials

Baicalein (98%) was purchased from Sigma-Aldrich. Silica gel (230-400 mesh, Sorbent Technologies Inc., Atlanta, GA) was used in open column chromatography (CC) fractionations. All other materials are described under Section 3.1.1.

4.1.2 Time course

Ten mg of baicalein was added into 4 mL of 5 M ammonium hydroxide and stirred at room temperature. Then 200 µL of reaction solution was taken out at 0, 5, 10, 20, 30, and 60 min, respectively. After incubation, 6 M hydrochloric acid was used to stop the reaction (final pH 5~6). Then the reaction solution was diluted by methanol 5 times. After centrifugation, the supernatant was taken out for HPLC-DAD analysis to check formation of the products. The peak areas of the products were compared to determine the best reaction time.

4.1.3 Large scale of reaction

To prepare enough reaction products, 100 mg of baicalein was added into 40 mL of 5 M ammonium hydroxide and stirred at room temperature. Half of the sample was taken after 20 min and the other half was taken after 60 min. 6 M hydrochloric acid was used to stop the

reaction (final pH 5~6). After acidification, the solution was centrifuged, and the supernatant and the sediment were checked by HPLC-DAD separately to confirm the formation of the products. The reaction solution was dried by rotary evaporator and reconstituted in 20:1 (methanol: water) and loaded into 80 g silica gel column, all elute was collected by auto-collector. The purification of the final aminated products was performed with Preparative-HPLC. The 20-min sample (50 mg) was used for the purification of products 1 and 2, and the 60-min sample (50 mg) was used for the purification of product 3.

4.1.4 HPLC-DAD analysis

Using the same HPLC-DAD instrument described under Section 3.1.4, we analyzed the formation of the reaction products of baicalein and ammonia. A Gemini 5μm C18 110A column (150 mm × 4.6 mm, 5μm; Phenomenex, Torrance, CA) was used to detect the reaction products. The mobile phases consisted of solvent A (95% deionized water and 5% methanol containing 0.1% formic acid) and solvent B (95% methanol and 5% water containing 0.1% formic acid). The gradient elution had the following profile: 5% B from 0 to 5 min; 5-100% B from 5 to 35 min; 100% B from 35 to 37 min; 100-0% B from 37 to 40 min; and 0% B from 40 to 45 min. The flow rate was 1 mL/min. The eluent was detected by HPLC-DAD with wavelength settings at 210, 275, 375, and 460 nm.

4.1.5 Silica gel column chromatography

Silica gel column was used to enrich the aminated products. In brief, column (2.0×40 cm) was packaged with 80 g of silica gel. The reaction products were dissolved in chloroform/methanol mixture (20:1) and loaded to the silica gel column. The column was eluted with chloroform/methanol mixture (20:1). The fractions were checked by HPLC-DAD and the fractions containing the aminated products were collected and further purified by preparative HPLC.

4.1.6 Preparative HPLC

The same preparative HPLC instrument described under Section 3.1.5 was used to separate the reaction products of baicalein and ammonia. The mobile phases consisted of solvent A (95% water and 5% methanol containing 0.1% formic acid) and solvent B (95% methanol and 5% water containing 0.1% formic acid). The gradient elution had the following profile: 5-100% B from 0 to 25 min; 100-5% B from 25 to 30 min; 5% B from 30 to 35 min. The flow rate was 3 mL/min. The eluent was detected by UV/Visible detector with wavelength settings at 210 and 275 nm and the peak containing aminated product was collected and dried by rotary evaporator. The purity of each aminated product was checked by analytical HPLC.

4.1.7 LC-MS analysis

The same LC-MS instrument described under Section 3.1.6 was used for the analysis of baicalein and its products. The negative ion polarity mode was set for an ESI ion source. The

instrument was tuned by baicalein and its products. Chromatographic separation was performed using a 150 mm × 3.0 mm i.d., 5 µm, Gemini C18 column (Phenomenex, Torrance, CA). The mobile phase consisted of mobile phase A (95% LC-MS grade water and 5% methanol with 0.1% formic acid) and mobile phase B (95% aqueous methanol with 0.1% formic acid). For the determination of the polyphenols, the gradient elution was performed for 45 min at a flow rate of 0.3 mL/min using the following gradient: 5% B from 0 to 5 min; 5-100% B from 5 to 35 min; 100% B from 35 to 37 min; 100-0% B from 37 to 40 min; and 0% B from 40 to 45 min. The injection volume was 10 µL.

4.1.8 NMR analysis

All purified compounds were dissolved in 200 µl of d 6-DMSO. ^{1}H-(600 MHz), ^{13}C-NMR (150 MHz), and all 2D NMR spectra including HMQC and HMBC were acquired on a Bruker Avance 600 MHz NMR instrument (Bruker Inc., Silberstreifen, Rheinstetten, Germany). Multiplicities were indicated by s (singlet), d (doublet), and m (multiplet). The ^{13}C-NMR spectra were proton decoupled.

4.1.9 Animal study

Two groups of mice (4 mice/group) were washed out for three days by feeding AIN-93G diet. After fasting overnight, one group was administered with baicalein (50 mg/mL in DMSO) by oral gavage at the dose of 200 mg/kg body weight. Another group was treated with the same volume vehicle (DMSO). After oral gavage, two groups of the mice were

moved into the metabolic cage separately to collect the urine and feces for 24 hours. The urine and stool were stored at -80 °C before analysis.

4.1.10 Preparation of the *in vivo* samples

Fecal and urine samples were prepared according to the methods described under Section 3.1.9.

4.2 Results

4.2.1 Time course

During the 60 min reaction, we monitored the formation of the aminated products at different time points. As shown in Figure 9, product 1 was detected at 5 min. At 10 min, most of the baicalein disappeared, and products 2 and 3 were generated. After 20 min, the amount of product 1 started to decrease, while the amount of product 3 dramatically increased. At 60 min, product 3 became the main product and product 1 disappeared. Based on this time course, 20 min was selected to prepare products 1 and 2 and 60 min for product 3.

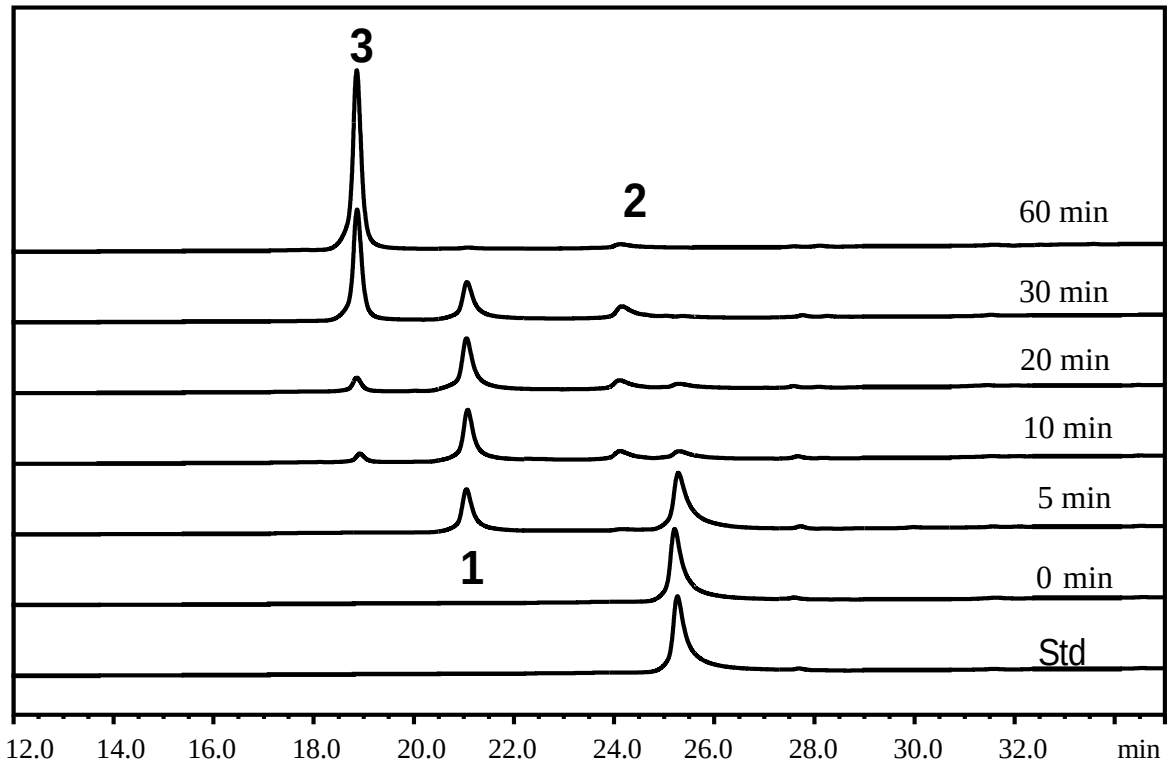

Figure 9. HPLC-DAD chromatograms of the reaction products of baicalein with 5 M of ammonium hydroxide at different reaction times.

4.2.2 Purification and characterization of the aminated product of baicalein

The reaction products were checked and confirmed by HPLC-DAD. The results showed that all of the supernatants and precipitants obtained from reactions for 20 min and 60 min contained three products (Figure 10). The three aminated products were purified by the combination of open column chromatography and preparative HPLC.

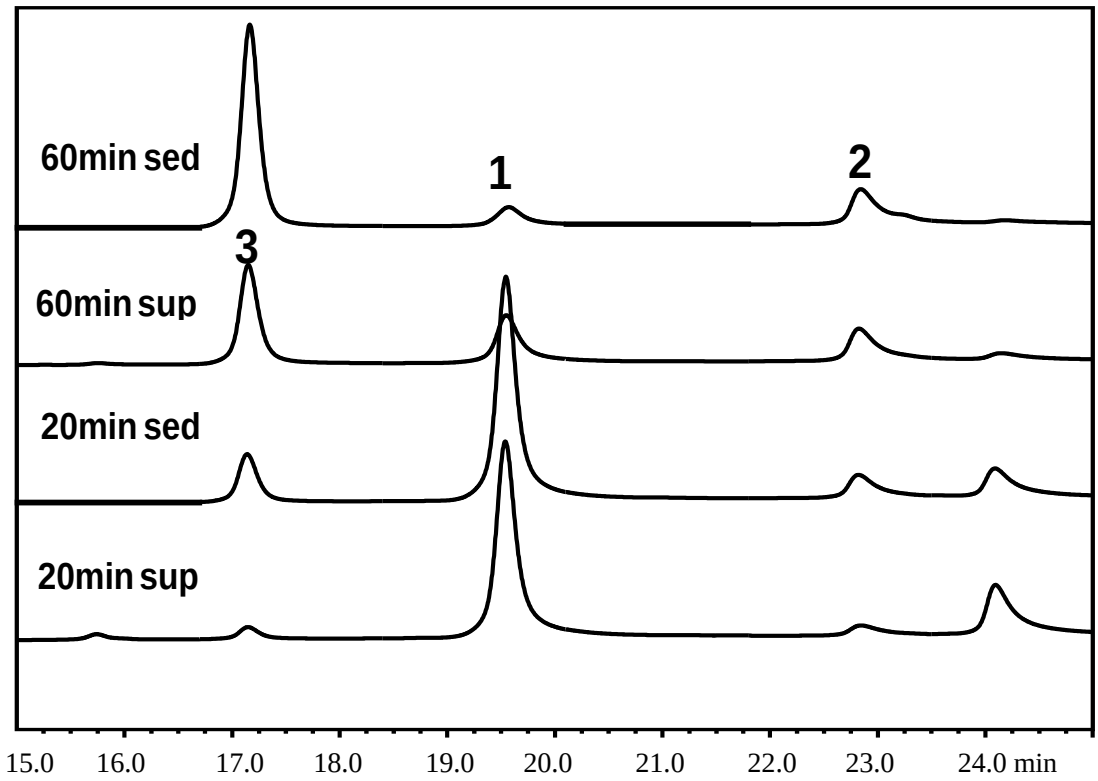

Figure 10. HPLC-DAD chromatograms of the supernatant (sup) and the sediment (sed) obtained from the large-scale reaction between baicalein and ammonium hydroxide for 20 and 60 min.

Products 1 and 2 had the same molecular weight of m/z 269 according to the molecular ions at m/z 268.1 $[M-H]^-$ from their negative ESI-MS spectra (Figure 11). This molecular weight was one Dalton less than that of baicalein, which indicated that one hydroxyl group of baicalein was replaced with amino group. Product 3 had a molecular weight of m/z 268 according to the molecular ion at m/z 267.1 $[M-H]^-$ under negative ESI- MS mode (Figure 11), suggesting two hydroxyl groups were replaced with two amino groups.

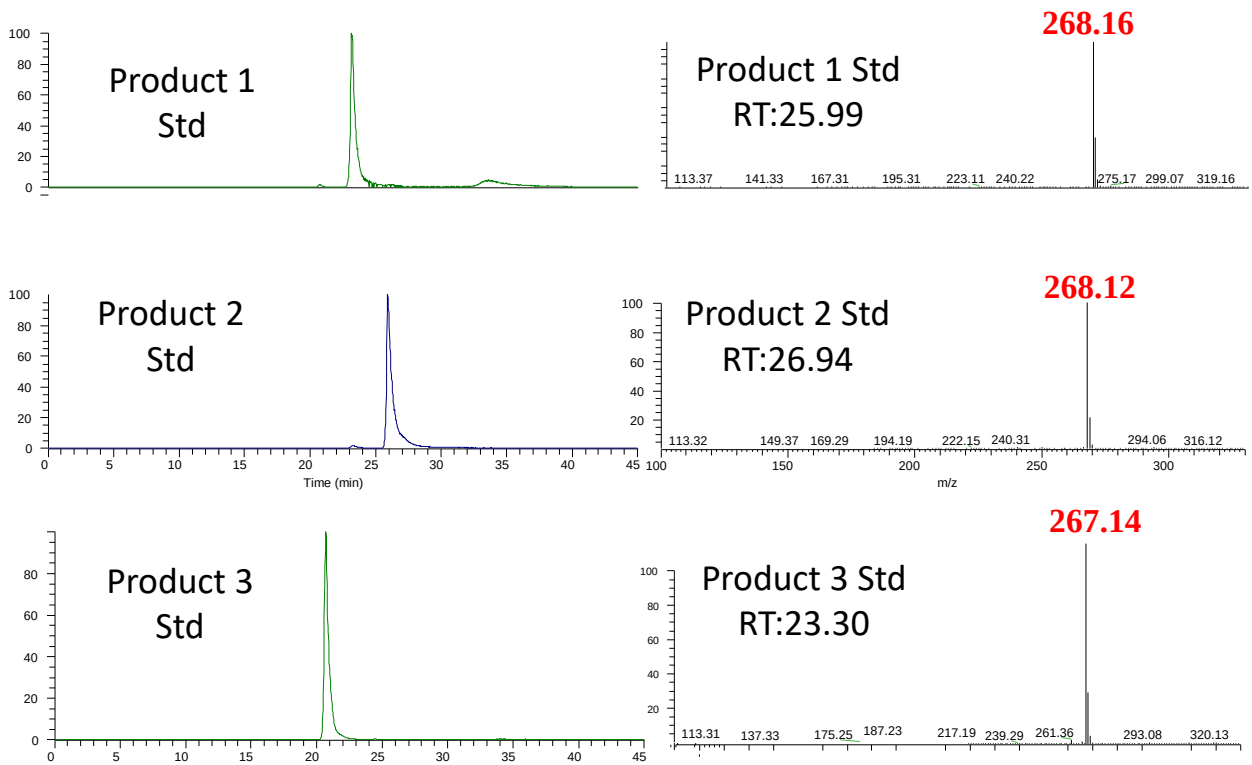

Figure 11. LC chromatograms and MS spectra of the three aminated products of baicalein

The ^{1}H-NMR spectrum of product 1 showed the unsubstituted B-ring signals at δ_H 8.04-8.05 (2H, m, H-2′,6′) and δ_H 7.55-7.59 (3H, m, H-3′,4′,5′) and the two singlet signals belonging to H-3 and H-8 which were the same to those of baicalein (Figure 12). Comparing with baicalein, the ^{13}C-NMR spectrum of product 1 showed signals for A-ring shifted downfield or upfield, but the signals for the B- and C-ring remained unchanged (Figure 13).

And its carbon signals were identical to those of 6-amino-5,7-dihydroxyflavone [41]. In order to confirm the position of amination, its 2D NMR spectra were recorded (Figures S9 and S10). The HMBC correlation from H-8 at δ_H 6.64 (s) to C-6 at δ_C 120.7 indicated that C-6 up shifted from δ_C 153.6 in baicalein to δ_C 120.7 in this aminated product. The HMBC correlation from H-3 at δ_H 6.89 (s) to C-5 at δ_C 144.3 as a 4*J* coupling (also denoted as a W-

coupling or M-coupling) indicated that C-5 up shifted from δ_C 147.0 in baicalein to δ_C 144.3 in this aminated product. The HMBC correlation from H-8 at δ_H 6.64 (s) to C-4 at δ_C 181.7 as a 4*J* coupling (also denoted as a W-coupling or M-coupling), to C-7 at δ_C 151.5 and C-9 at δ_C 148.6 indicated that C-9 down shifted from δ_C 129.2 in baicalein to δ_C 148.6 in this aminated product (Figure 14). Based on the analysis of its ^{1}H, ^{13}C, HMQC, and HMBC NMR data, its protons and carbons were assigned in Table 2. Thus, its structure was characterized as 6-amino-5,7-dihydroxylflavone, named as 6-NH_2-baicalein (Figure 14).

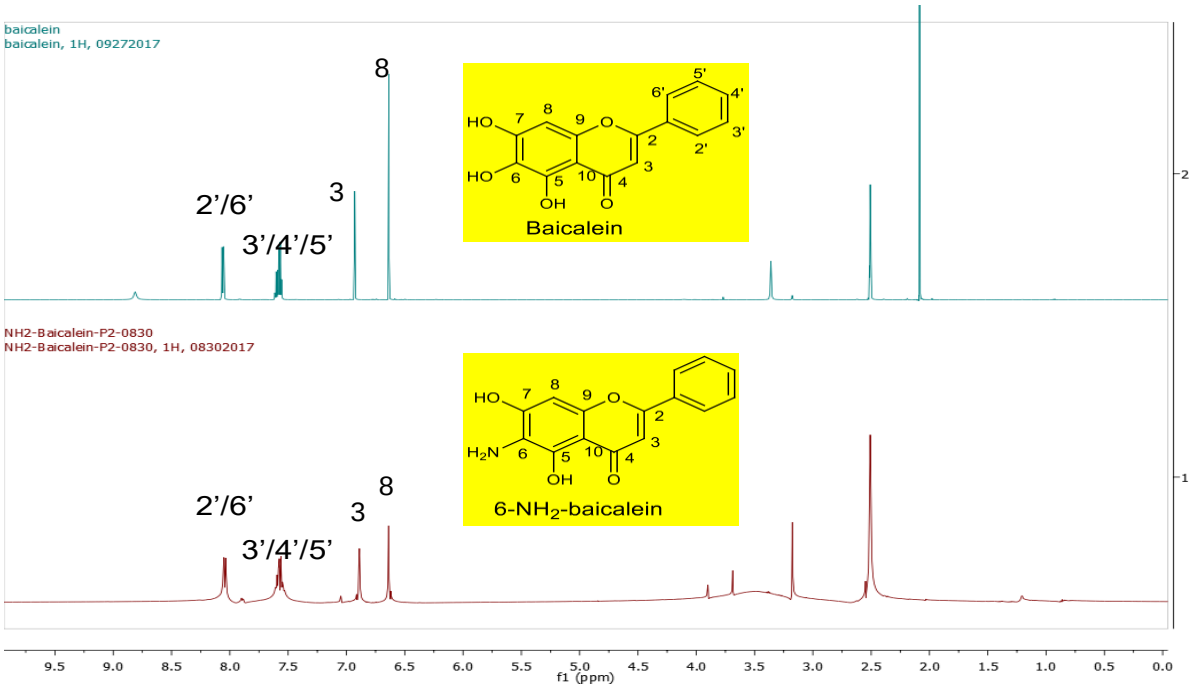

Figure 12. The ^{1}H-NMR spectra of product 1 (6-NH_2-baicalein) and baicalein in d_6-DMSO

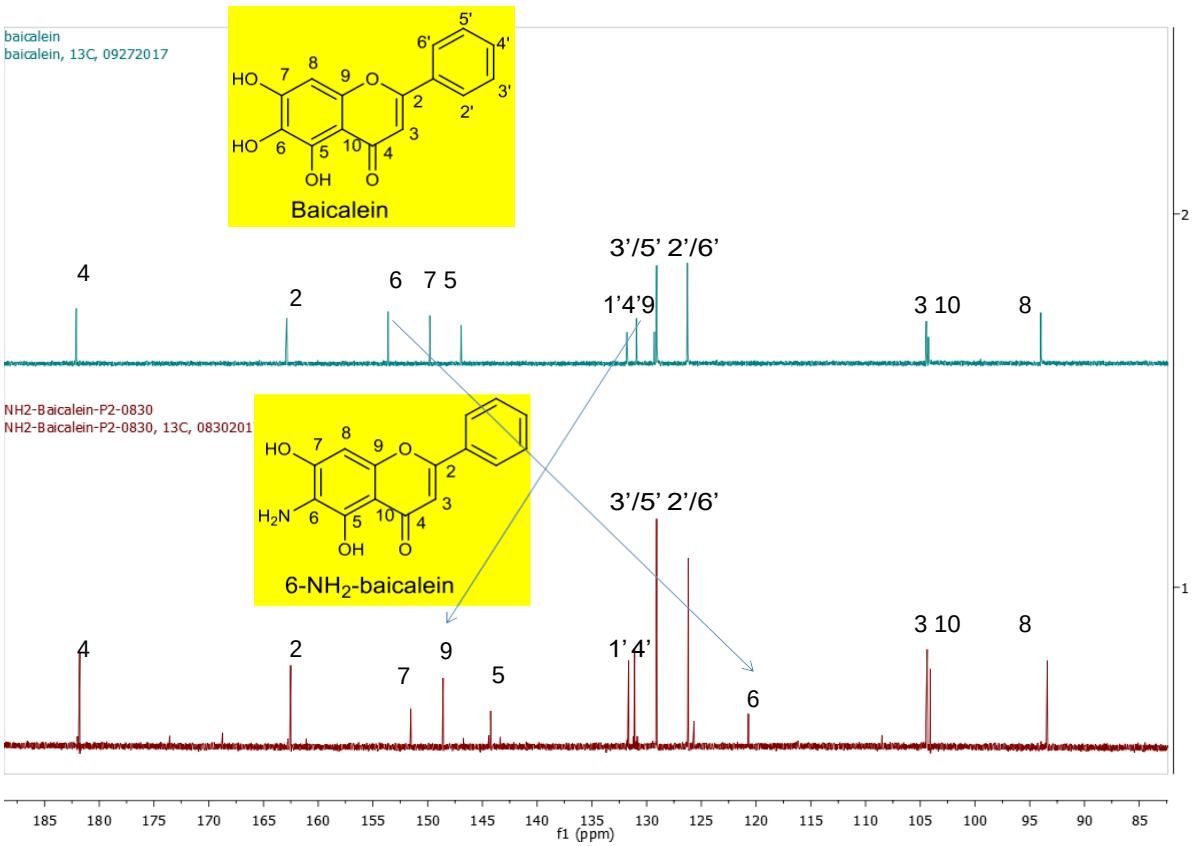

Figure 13. The ^{13}C-NMR spectra of product 1 (6-NH_2-baicalein) and baicalein in d_6-DMSO

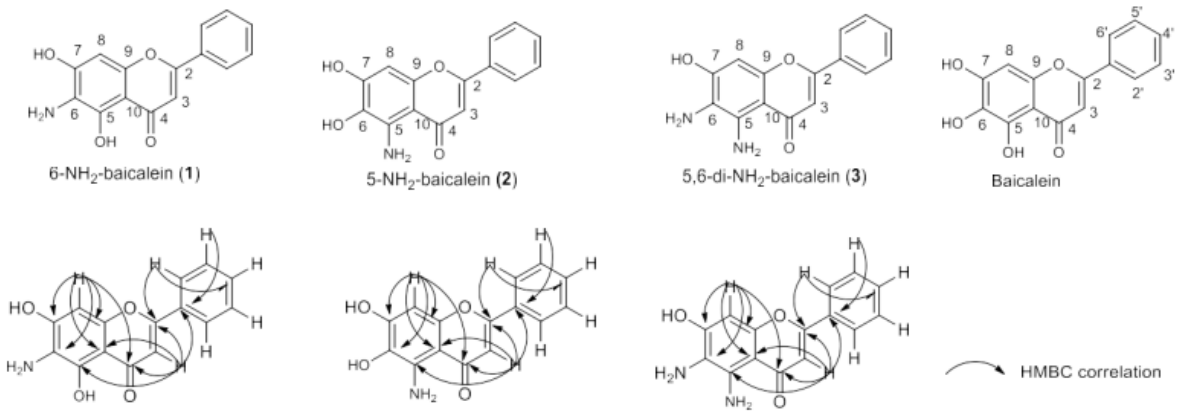

Figure 14. The structures of baicalein and its three aminated products (1, 2, 3) and their key HMBC correlations.

Product 2 showed a very similar proton spectrum with that of baicalein as well as product 1 including the unsubstituted B-ring signals at δ_H 7.98-7.99 (2H, m, H-2´,6´) and δ_H

7.54-7.57 (3H, m, H-3´,4´,5´) and the two singlet signals at δ_H 6.30 (s) and 6.65 (s) belonging to H-3 and H-8. The HMBC correlations from H-8 at δ_H 6.30 (s) to C-6 at δ_C 126.3 and H-3 at δ_H 6.65 (s) to C-5 at δ_C 139.1 as a 4*J* coupling (also denoted as a W-coupling or M-coupling) showed that C-5 up shifted further in product 2 (δ_C 139.1) than in product 1 (144.1). Comparing with product 1, the chemical shift of C-6 slightly down shifted to 126.3 ppm from 120.7 All of these spectral features indicated that the amination happened at C-5 in product 2 (Figure 15). Based on the analysis of its ^{1}H, ^{13}C, HMQC, and HMBC NMR data, its protons and carbons were assigned in Table 2. Its structure was elucidated as 5-amino-6,7- dihydroxylflavone, named as 5-NH_2-baicalein (Figure 14).

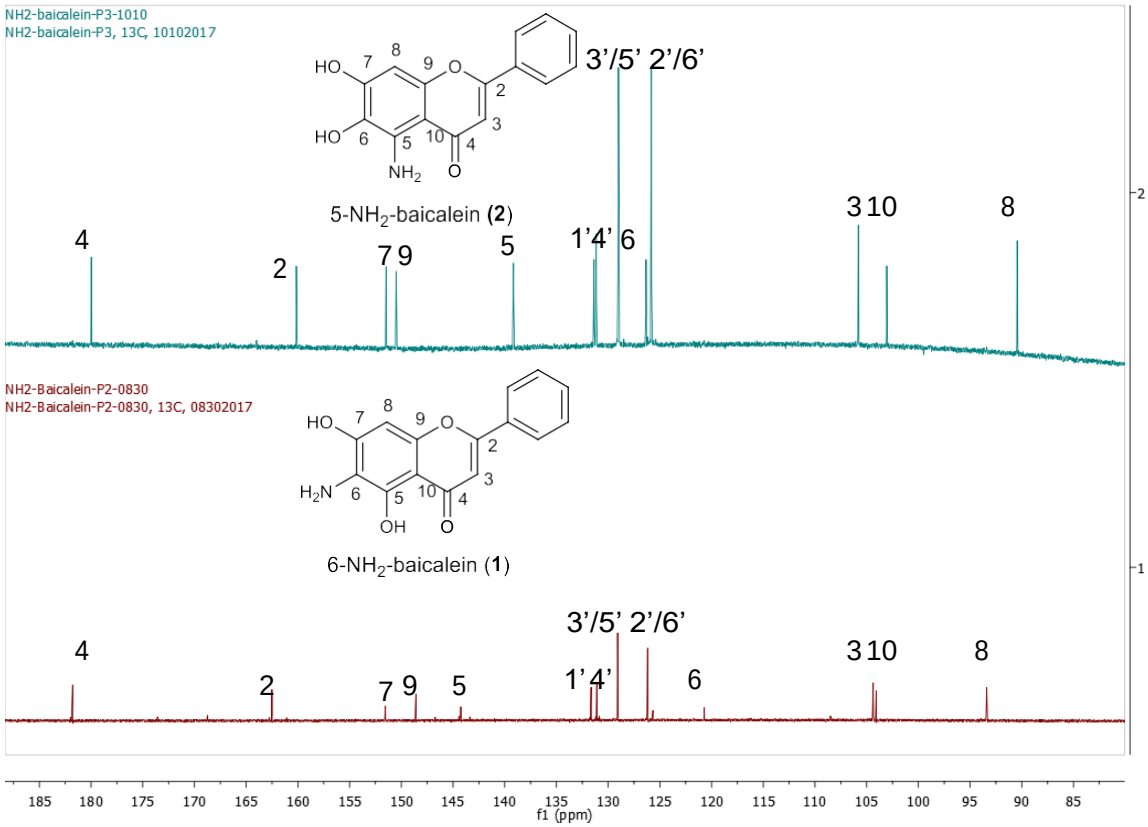

Figure 15. The ^{13}C-NMR spectra of products 1 and 2

The molecular weight of product 3 was two mass units less than that of baicalein (Figure 12), suggesting two hydroxyl groups of baicalein were possibly replaced with two amino groups. This was supported by the observation that product 3 was generated at a later time point after the formation of product 1 and peaked at 60 min, and product 1 decreased and disappeared at 60 min (Figure 10). Therefore, we speculated that product 3 was the 5, 6-di-NH_2-baicalein, which was further confirmed by the analysis of its 1D and 2D NMR data. The ^{1}H-NMR spectrum of product 3 also showed the unsubstituted B-ring signals at δ_H 7.96 (2H, m, H-2′,6′) and δ_H 7.53 (3H, m, H-3′,4′,5′) and the two singlet signals at δ_H 6.65 and 6.40 belonging to H-3 and H-8 (Table 2). Comparing with baicalein and product 1, the carbon NMR spectrum of product 3 showed a dramatic upfield shift for both C-6 and C-5, C-6 in 3 shifted up to 117.3 ppm from 153.6 in baicalein and C-5 shifted up to 136.6 ppm in 3 from 147.0 in baicalein. The HMBC correlations from H-3 at δ_H 6.65 to C-5 at δ_C 136.6, H-8 at δ_H 6.40 to C-6 at δ_C 117.3 confirmed that both hydroxyl groups on C-5 and C-6 were replaced with amino groups. Therefore, product 3 was confirmed as 5, 6-diamino-7-hydroxylflavone, named as 5, 6-di-NH_2-baicalein (Figure 14). Its proton and carbon signals were assigned based on the analysis of its 1D and 2D NMR data and were shown in Table 2.

Table 2. ^{1}H-(600 MHz) and ^{13}C-NMR (150 MHz) data of aminated myricetin products (1, 2,3) (in d6-DMSO, δ in ppm, J in Hz)

No.	δ_H				δ_C		
	1	2	3		1	2	3
2	-	-	-		162.5	160.1	159.8
3	6.89 (s)	6.65 (s)	6.65 (s)		104.4	105.8	105.9
4	-	-	-		181.7	180.1	180.1
5	-	-	-		144.3	139.1	136.6
6	-	-	-		120.7	126.3	117.3
7	-	-	-		151.5	151.4	150.7
8	6.64 (s)	6.30 (s)	6.40 (s)		93.4	90.4	90.8
9	-	-	-		148.6	150.5	149.5
10	-	-	-		104.0	103.1	103.3
1´	-	-	-		131.6	131.3	131.4
2´	7.55-7.59 (m)	7.98-7.99 (m)	7.96 (brs)		126.2	125.8	125.7
3´	8.04-8.05 (m)	7.54-7.57 (m)	7.53 (brs)		129.0	129.0	129.0
4´	8.04-8.05 (m)	7.54-7.57 (m)	7.53 (brs)		131.1	131.1	131.0
5´	8.04-8.05 (m)	7.54-7.57 (m)	7.53 (brs)		129.0	129.0	129.0
6´	7.55-7.59 (m)	7.98-7.99 (m)	7.96 (brs)		126.2	125.8	125.7

4.2.3 Animal study

The existence of the aminated baicalein products were searched for in the urine and fecal samples collected from baicalein-treated CF1 mice using LC-MS/MS and their

retention times and tandem mass spectra were compared with those of the synthetic products 1-3. No aminated baicalein was found in baicalein-treated urine sample. However, in baicalein-treated mouse fecal samples, two clear peaks were shown under the search of mono-aminated baicalein (Figure 16). Their retention times and tandem mass spectra were almost identical to those of products 1 and 2 (Figure 16), indicating that baicalein could be oxygenized to ortho-quinone and further react with ammonia produced in the GI tract through nucleophilic addition to generate the aminated metabolites. No di-aminated baicalein metabolite was detected in either urine or fecal samples.

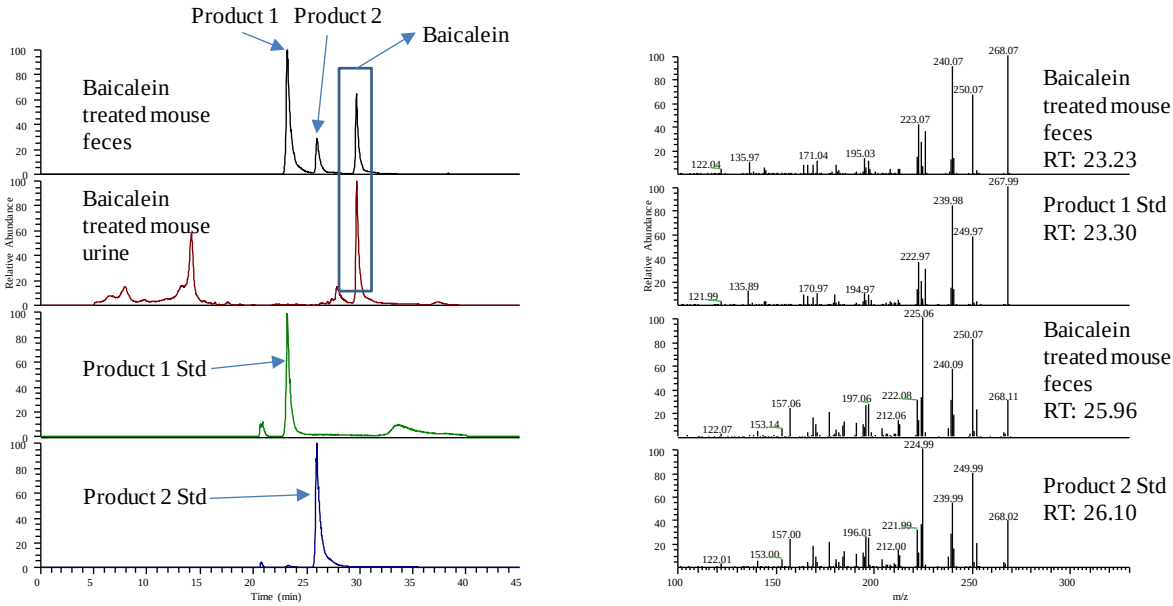

Figure 16. LC chromatograms of the standards of products 1 and 2 and the peaks in mouse fecal samples under selected ion monitoring (SIM) mode and their corresponding MS/MS (negative ion) spectra.

4.3 Conclusion

From the chemical reaction, baicalein could be aminated very quickly and the amination happened on C-6 of A-ring first, and then on C-5 of A-ring to produce the mono-NH_2-baicalein with 6-NH_2-baicalein being the most abundant one. The mono-NH_2-baicalein could be further aminated to generate the 5, 6-di-NH_2-baicalein, which is the dominant product after 60 min of reaction. Further *in vivo* studies identified the two mono-NH_2-baicalein products as the major novel metabolites of baicalein in mice. The study on baicalein provides the evidence that a flavonoid with a *vic*-trihydroxy structure on A-ring is also the substrate for animation both *in vitro* and *in vivo*.

Discussion

Many studies have shown that dietary flavonoids, which are abundant in the human diet in foods such as onions, berries, tea, citrus fruits, and red wine, have significant antioxidative properties and may prevent cancer and cardiovascular diseases. The widespread distribution and relatively low toxicity of dietary flavonoids also played an important role in increasing interest among researchers and manufacturers. However, there is still a wide gap in the understanding of flavonoid activities and their practical application due to the low bioavailability of most dietary flavonoids and our deficient knowledge of the *in vivo* biotransformation mechanism of these dietary flavonoids. Previous study has demonstrated that tea catechins, such as EGCG and EGC, have the capacity to remove ammonia in water to generate the aminated product, in which the amine replaced the middle hydroxyl group of the *vic*-trihydroxyl group on B ring. In this study, we presume myricetin, which also contains a pyrogallol nucleus on the B ring, may undergo a similar aminated metabolic pathway. Our animal study and spectroscopic analysis including LC-MS/MS and NMR confirmed this hypothesis. As we expected, the amination occurred at the C-4 position on the B-ring of myricetin. Furthermore, we speculate baicalein, a flavone with a *vic*-trihydroxyl group on the A ring, may also undergo a similar aminated pathway. Interestingly, baicalein was found to form three aminated products and two mono aminated metabolites were confirmed *in vivo*. Additionally, the aminated products of baicalein are the

major metabolites *in vivo*.

There is a great diversity of the impact of our study. Amine is a reactive functional group, and the replacement of a hydroxyl group with an amino group may improve the bioactivities of dietary flavonoids. With this new function group, the aminated flavonoids could conjugate with the endogenous molecules such as reactive carbonyl species to improve our health condition. In addition, this novel metabolic pathway advances our knowledge on the biotransformation of dietary flavonoids. The low bioavailability of dietary flavonoids is a deep gap that obstructs our health benefits *in vivo*. We might underestimate the total levels of flavonoids and their metabolites and the contribution of the metabolites of flavonoids to their health effects. For example, we identified 5- and 6-NH_2-baicalein as the major metabolites of baicalein in mice, which are even more abundant than baicalein. These novel metabolites may provide significant contributions to the observed *in vivo* efficacy of baicalein.

In this study, we confirmed that amination is a newly discovered metabolic pathway for myricetin and baicalein which indicates these flavonoids can react with ammonia *in vivo* to produce the aminated metabolites. The ammonia in the human body is mostly generated from the gut, which is probably the reason for finding high levels of these metabolites in mouse fecal samples. In the small intestine, with the enzyme k-phosphate-activated glutaminase (k-PAG), enterocytes convert glutamine to glutamate. This produces energy, as well as nucleotides and ammonia. In the colon, the majority of ammonia is generated from the breakdown of urea by intestinal flora-derived urease [36]. Ammonia is primarily a waste product of cellular metabolism and produces harmful properties at higher concentrations.

Systemic hyperammonemia has been largely found in patients with cirrhosis and hepatic encephalopathy [38]. Moreover, increased ammonia in the brain has been linked to astrocyte edema and impairment in the neurotransmission that leads to hepatic encephalopathy [38]. Our study showed the potential ability of flavonoids to trap ammonia with a *vic*-trihydroxyl structure either on the A-ring or B-ring, which may have an impact on blocking ammonia absorption for the gut in the human body, related to the high-ammonia diseases. Further study in this field is needed, as well as additional exploration of *in vivo* ammonia removal by dietary flavonoids to confirm that taking flavonoids may be a plausible treatment method for hyperammonemia. Furthermore, measurements of the activity from aminated metabolites will help us to have a better understanding of the *in vivo* efficacy of dietary flavonoids.

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Appendix

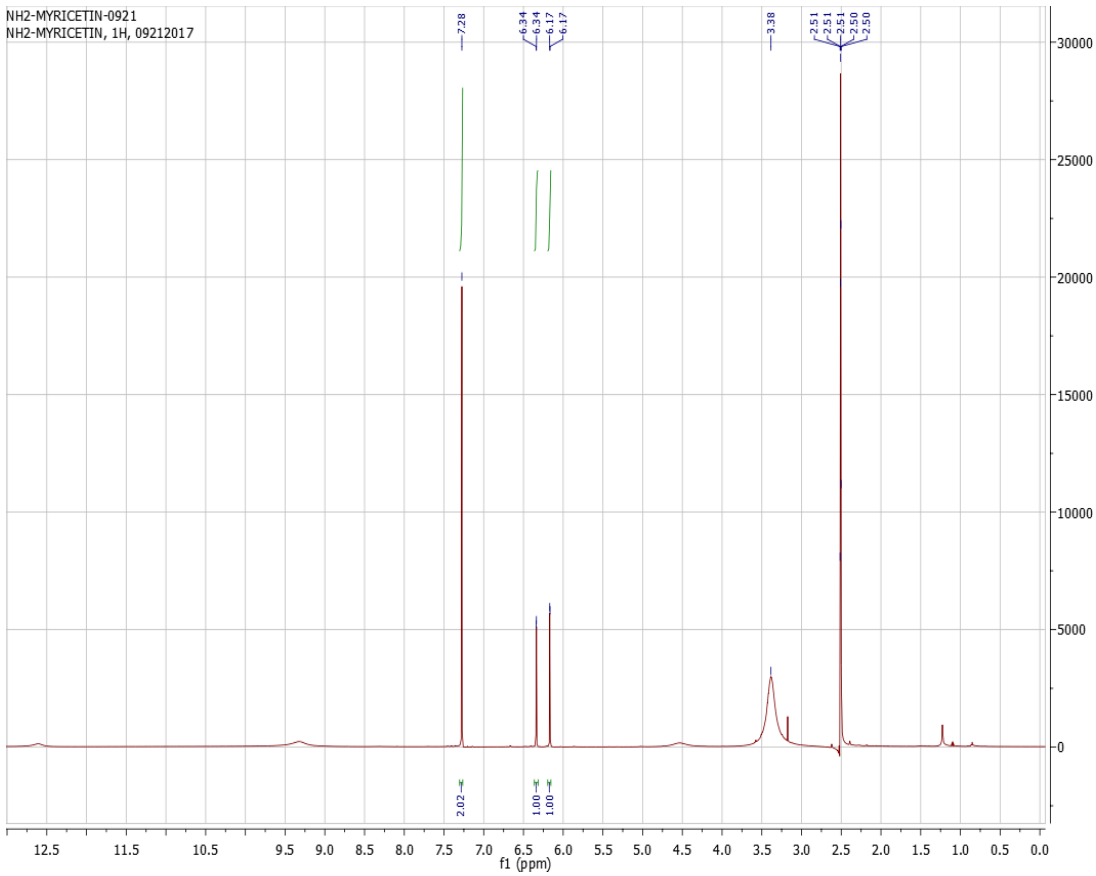

Figure S1. ^{1}H-NMR spectrum of 4´-NH_2-myricetin in d_6-DMSO.

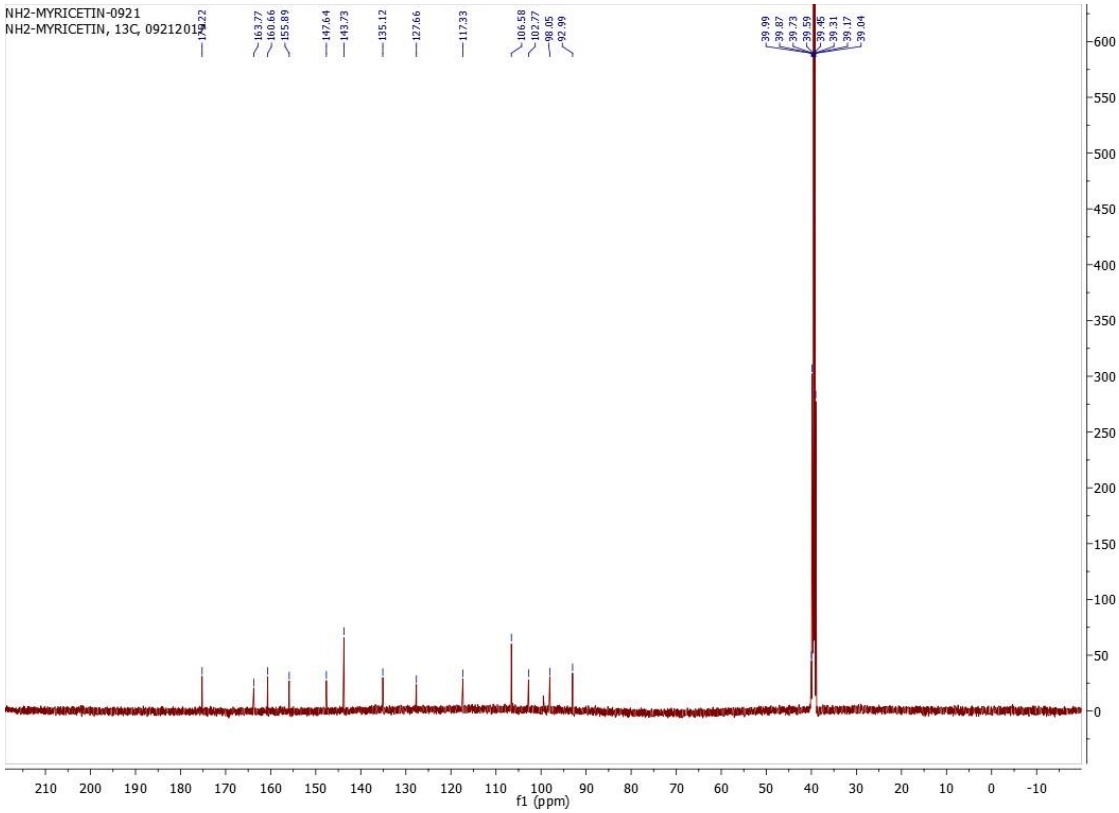

Figure S2. ^{13}C-NMR spectrum of 4´-NH_2-myricetin in d6-DMSO.

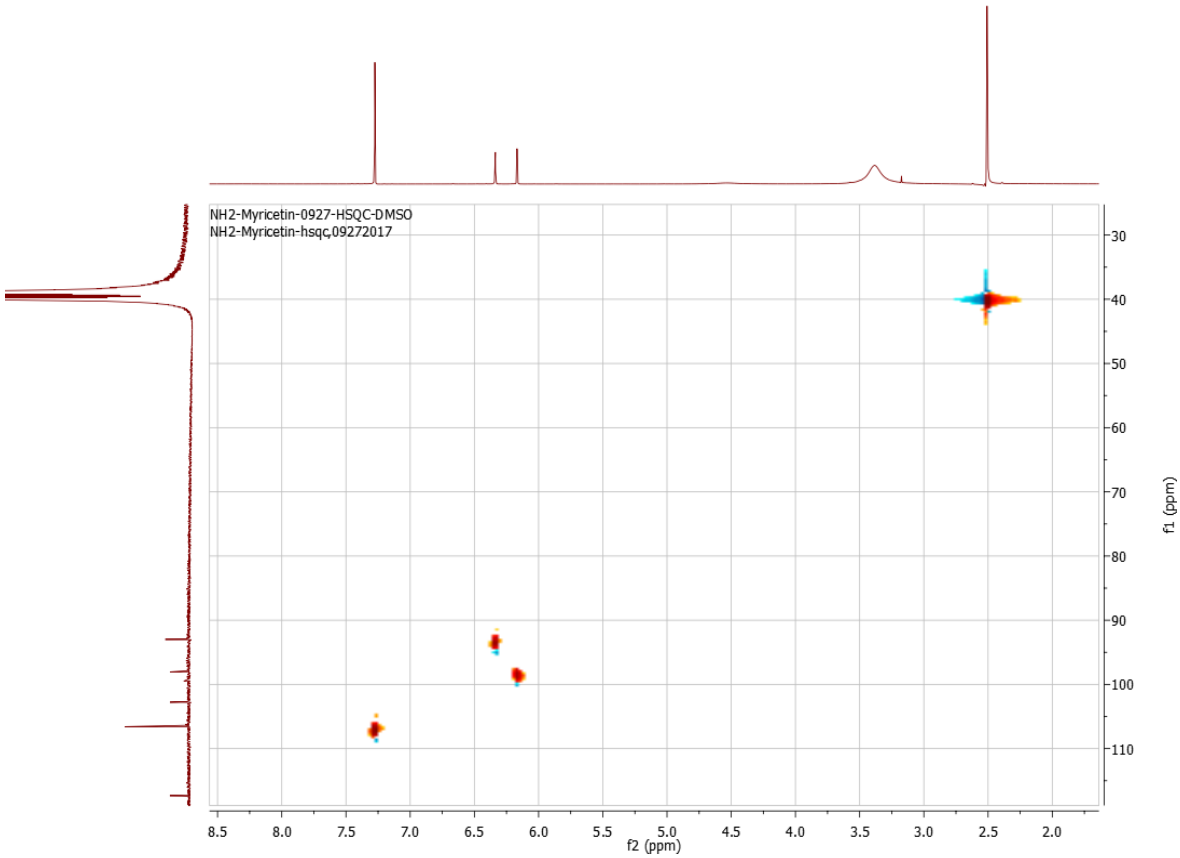

Figure S3. HMQC spectrum of 4´-NH_2-myricetin in d6-DMSO.

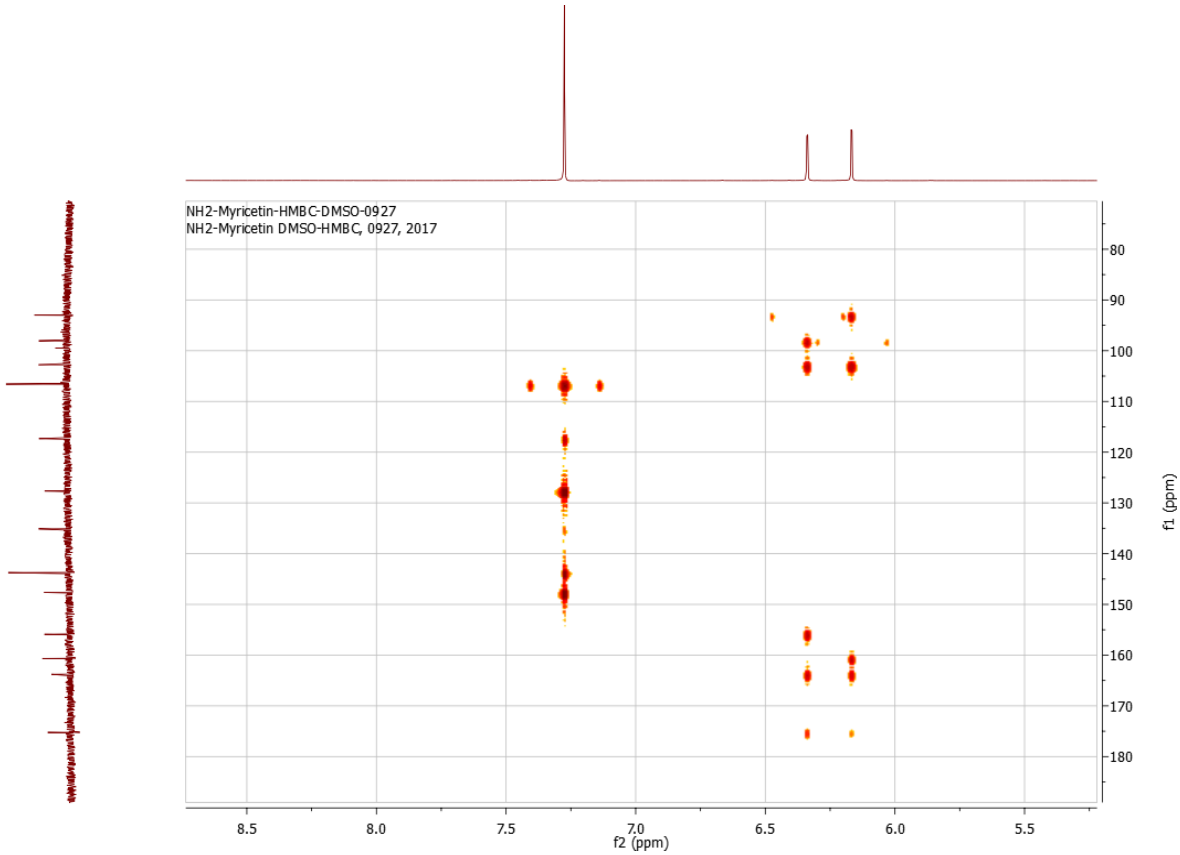

Figure S4. HMBC spectrum of 4´-NH_2-myricetin in d6-DMSO.

Figure S5. ^{1}H-NMR spectrum of myricetin in d_6-DMSO.

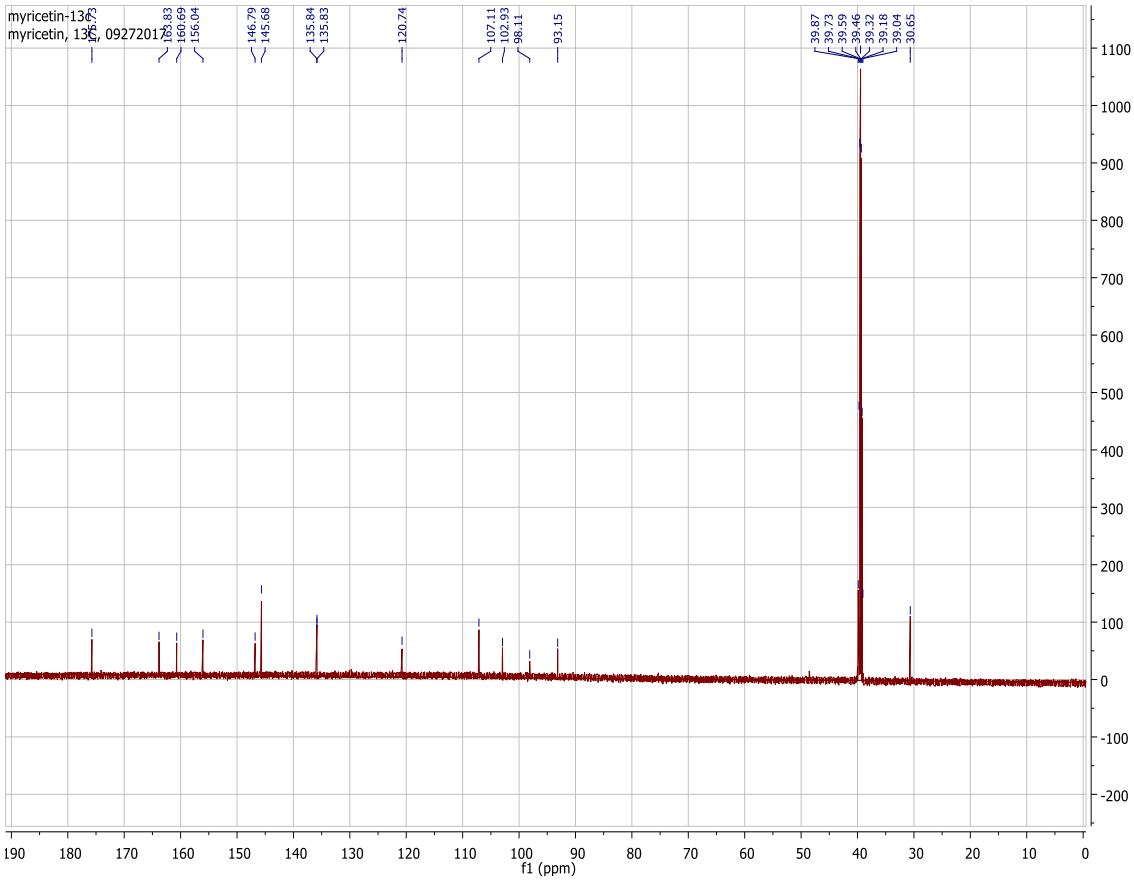

Figure S6. ^{13}C-NMR spectrum of myricetin in d_6-DMSO.

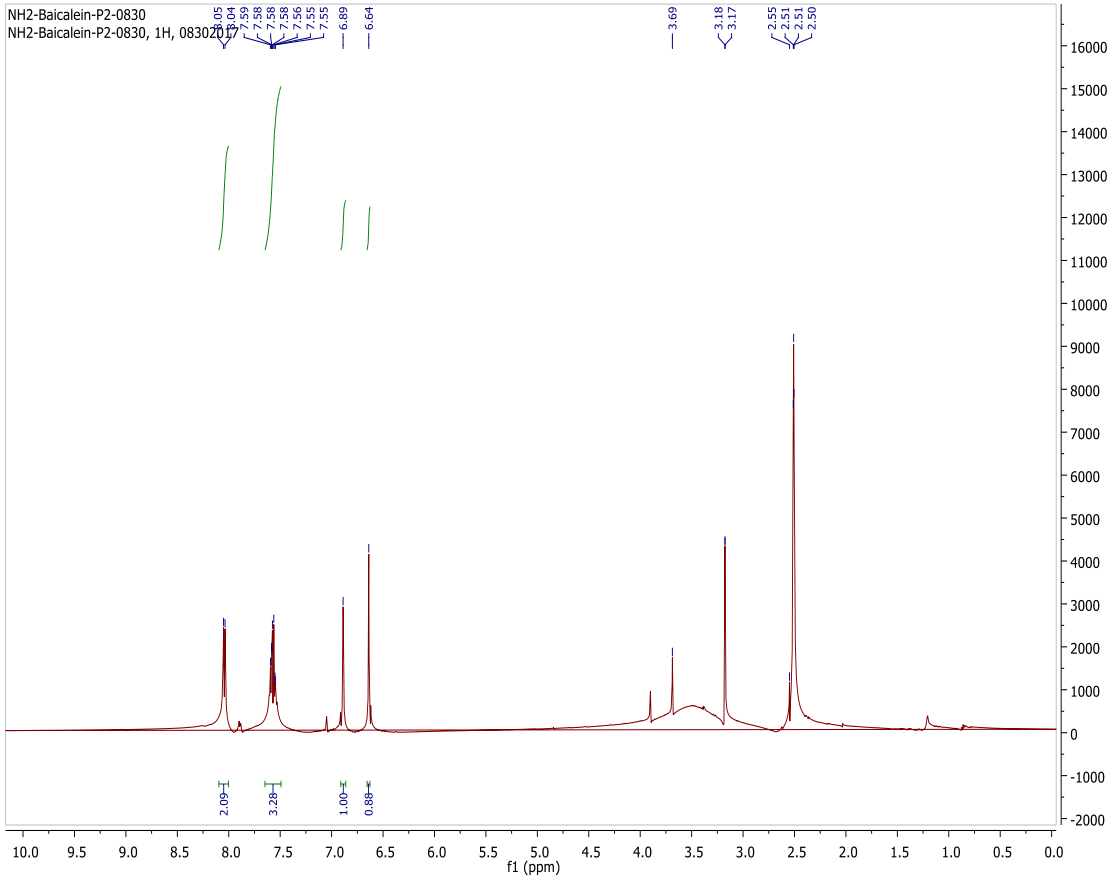

Figure S7. ^{1}H-NMR spectrum of of 6-NH_2-baicalein (1) in d6-DMSO.

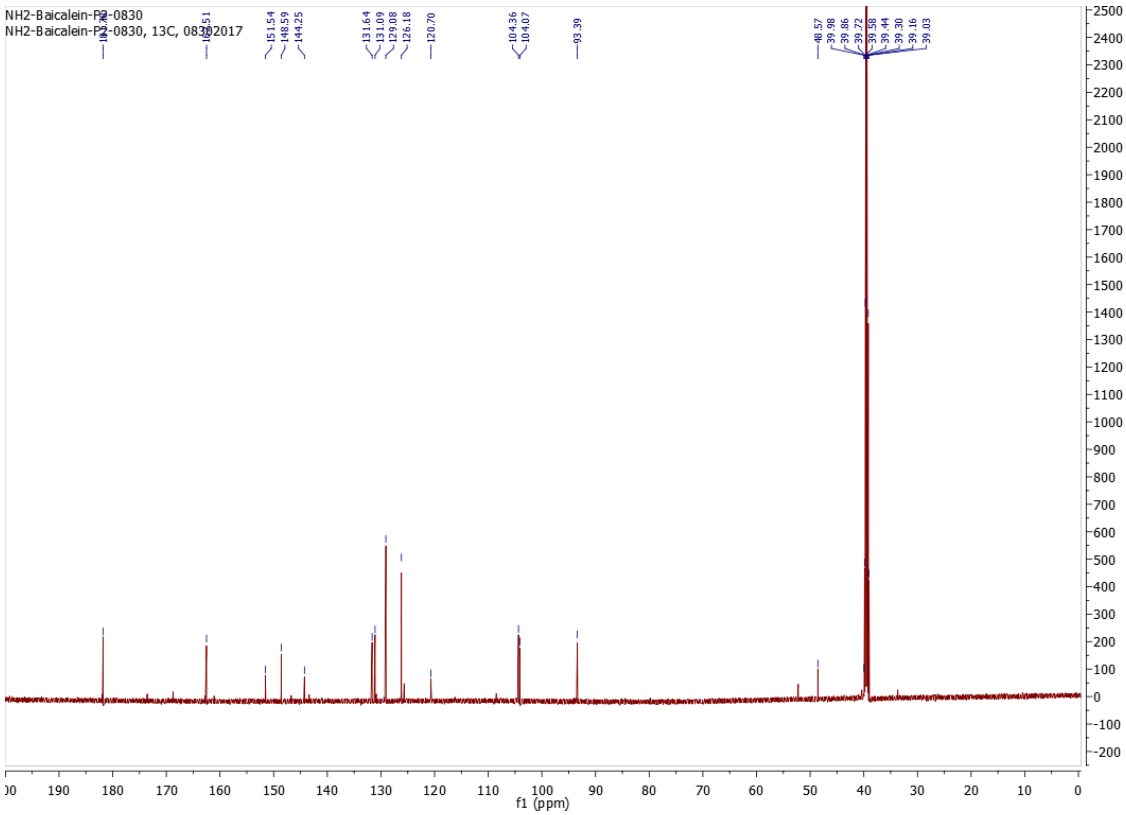

Figure S8. ^{13}C-NMR spectrum of of 6-NH_2-baicalein (1) in d6-DMSO.

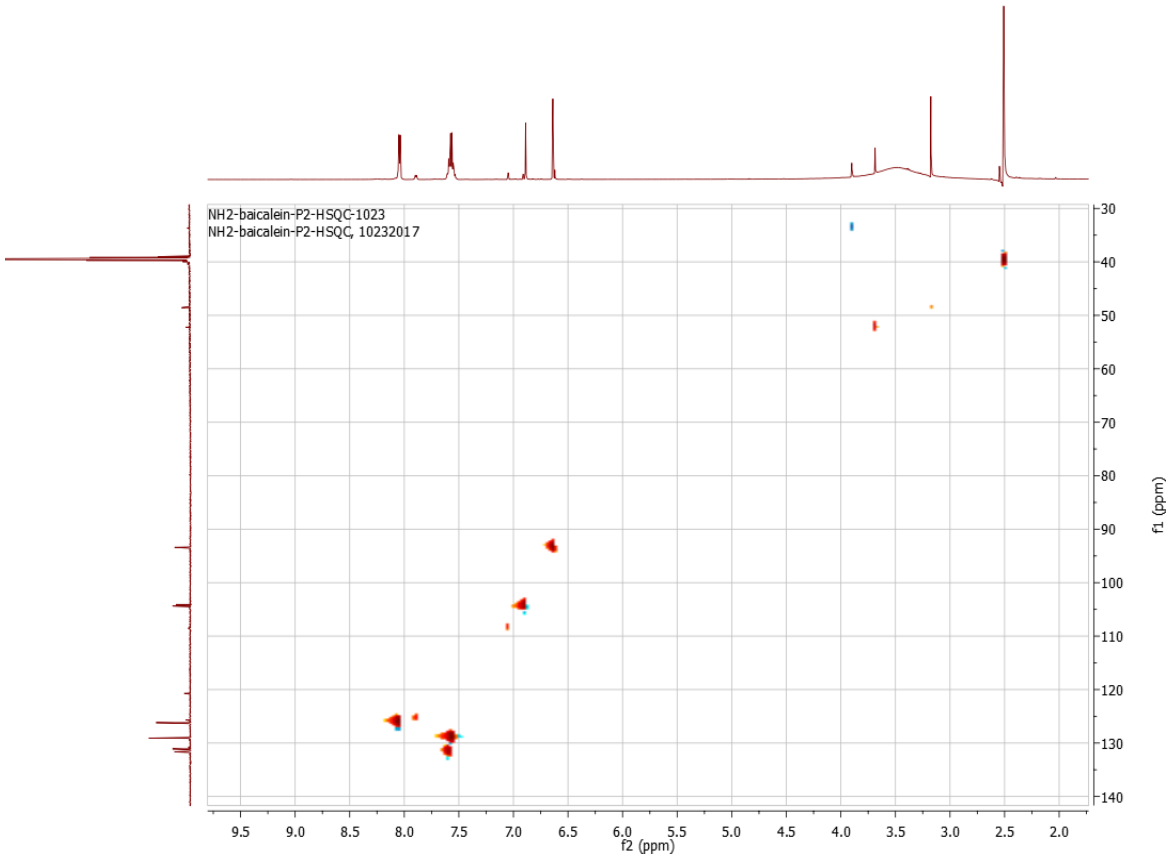

Figure S9. HMQC spectrum of 6-NH_2-baicalein (1) in d_6-DMSO.

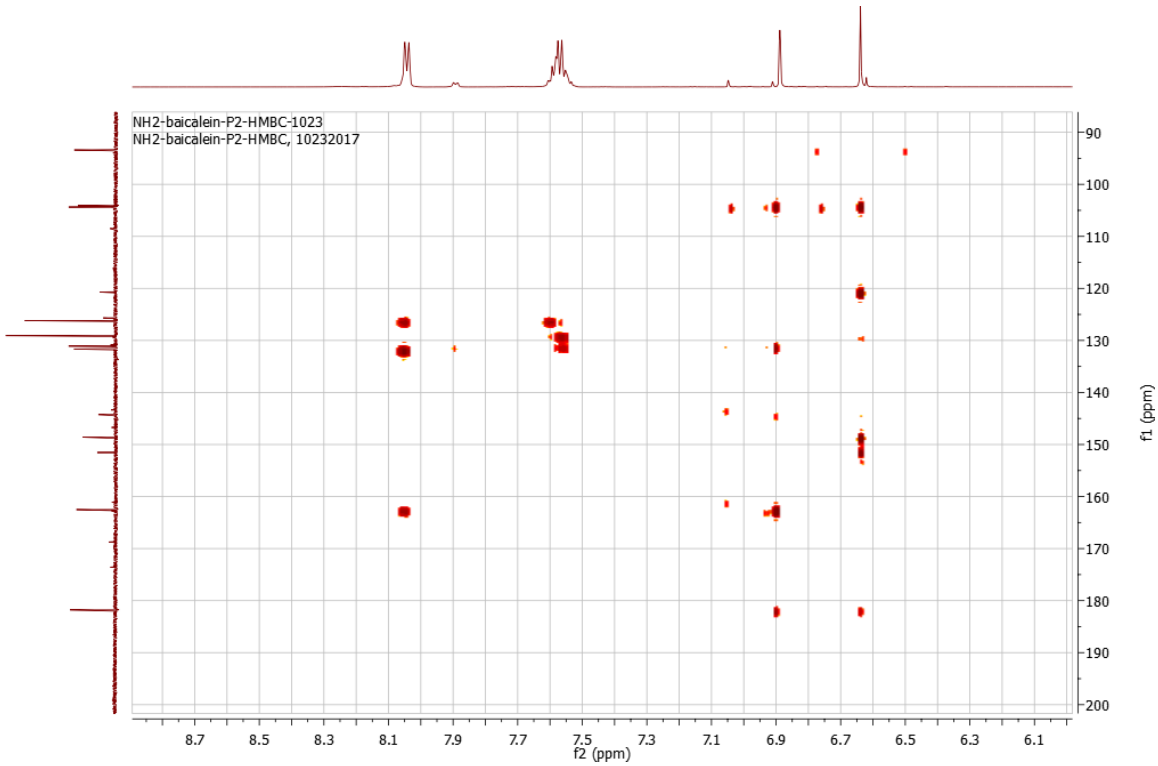

Figure S10. HMBC spectrum of 6-NH_2-baicalein (1) in d_6-DMSO.

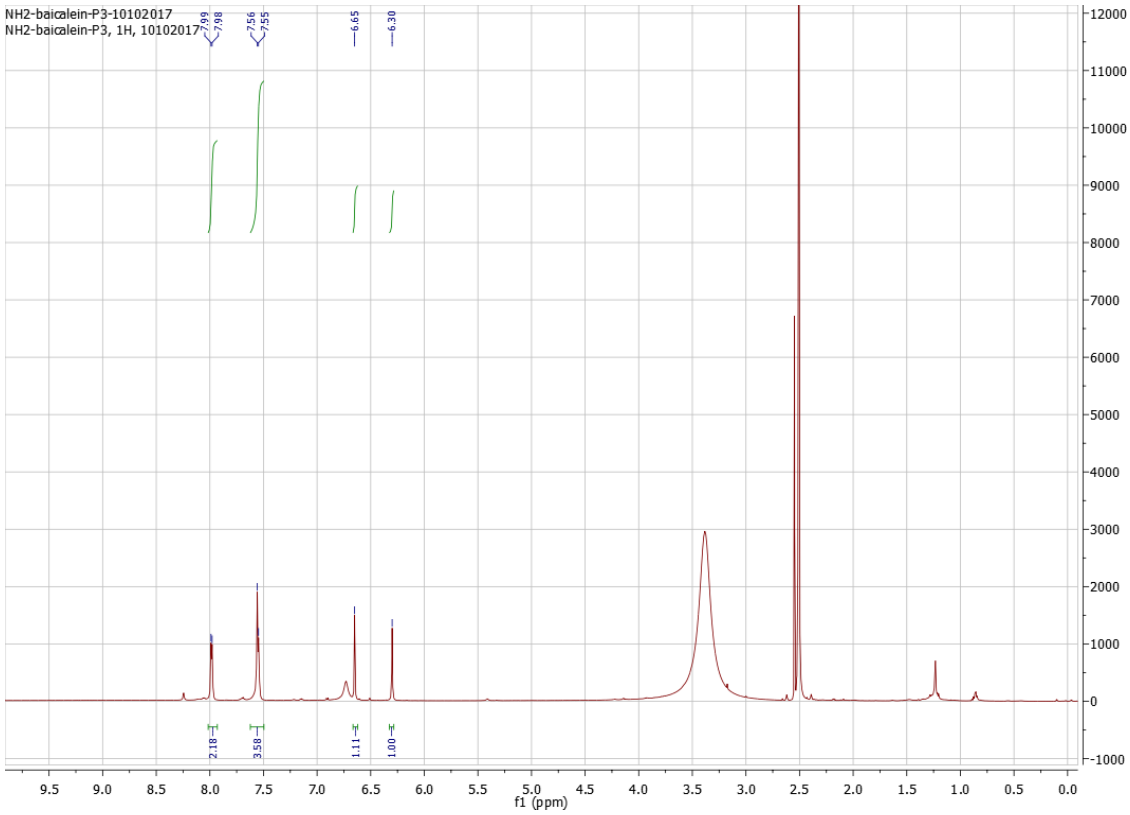

Figure S11. ^{1}H-NMR spectrum of 5-NH_2-baicalein (2) in d6-DMSO.

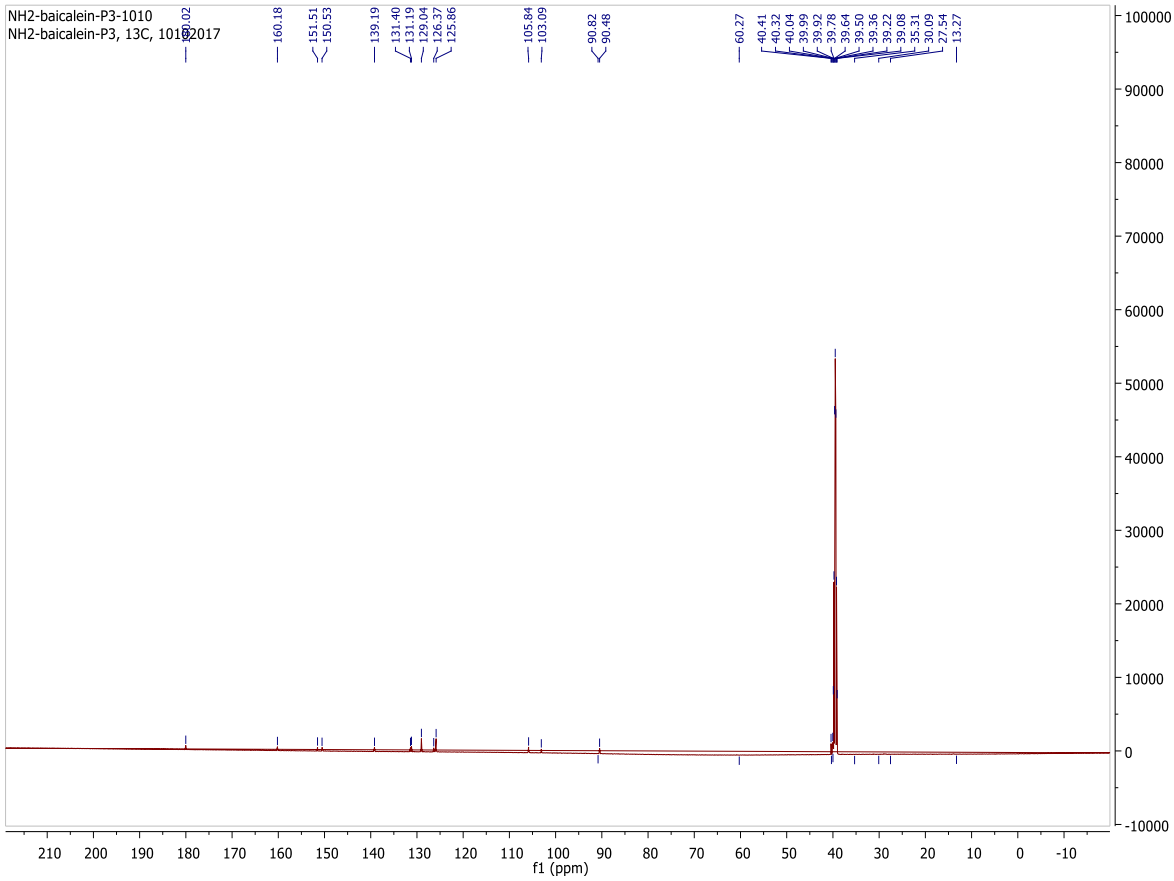

Figure S12. ^{13}C-NMR spectrum of 5-NH_2-baicalein (2) in d6-DMSO.

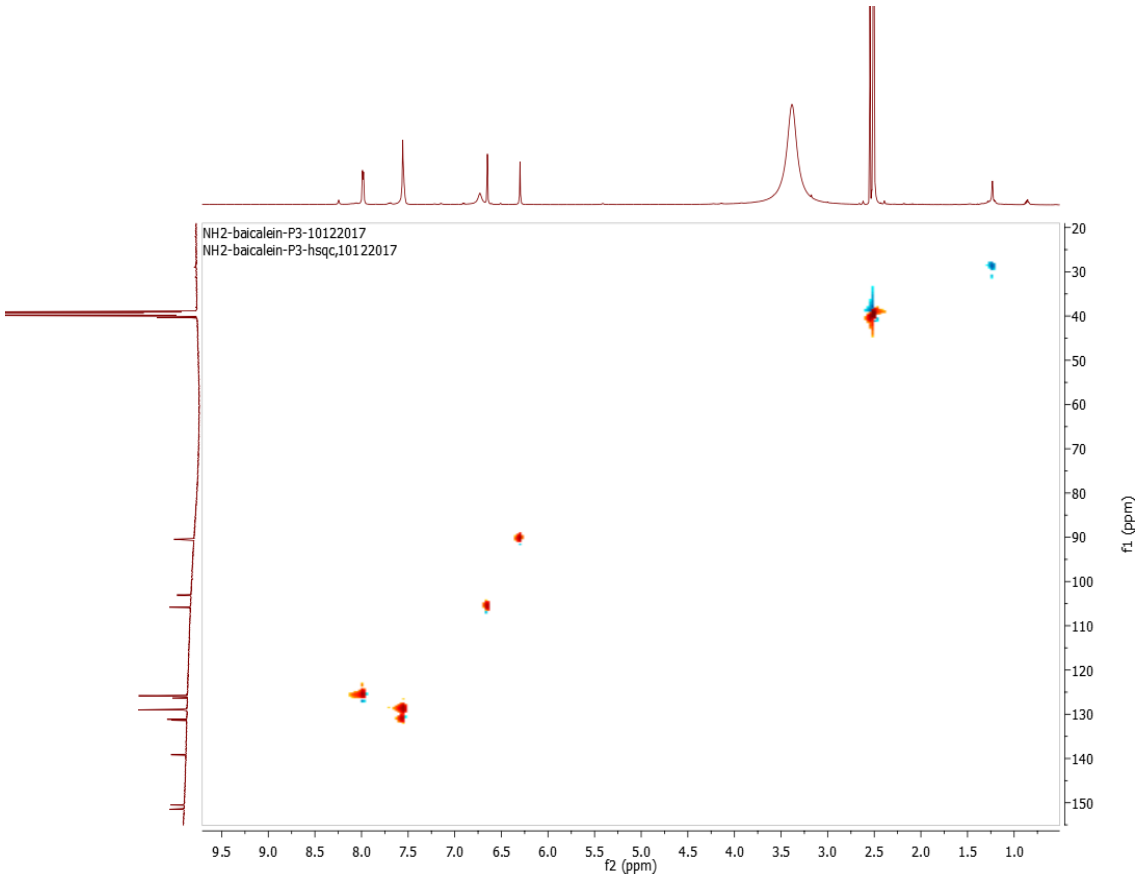

Figure S13. HMQC spectra of 5-NH_2-baicalein (2) in d_6-DMSO.

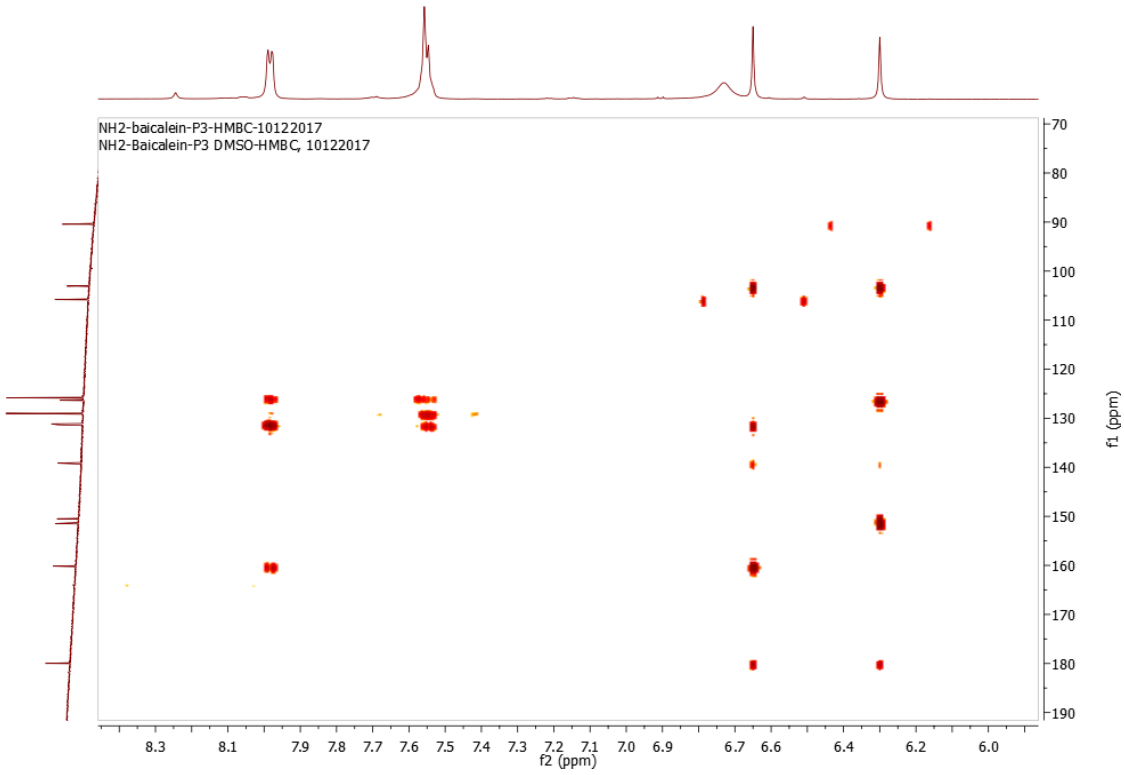

Figure S14. HMBC spectra of 5-NH_2-baicalein (2) in d_6-DMSO.

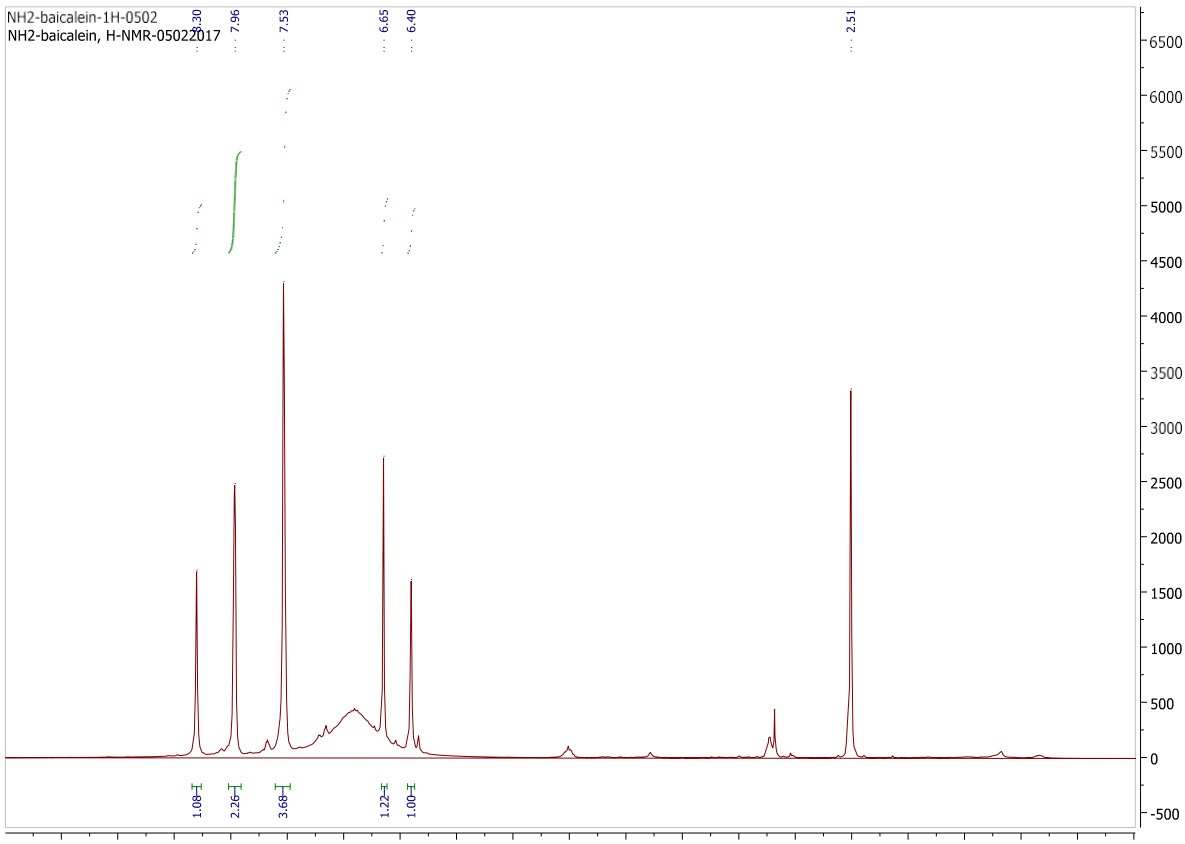

Figure S15. ^{1}H-NMR spectra of 5, 6-di-NH_2-baicalein (3) in d_6-DMSO.

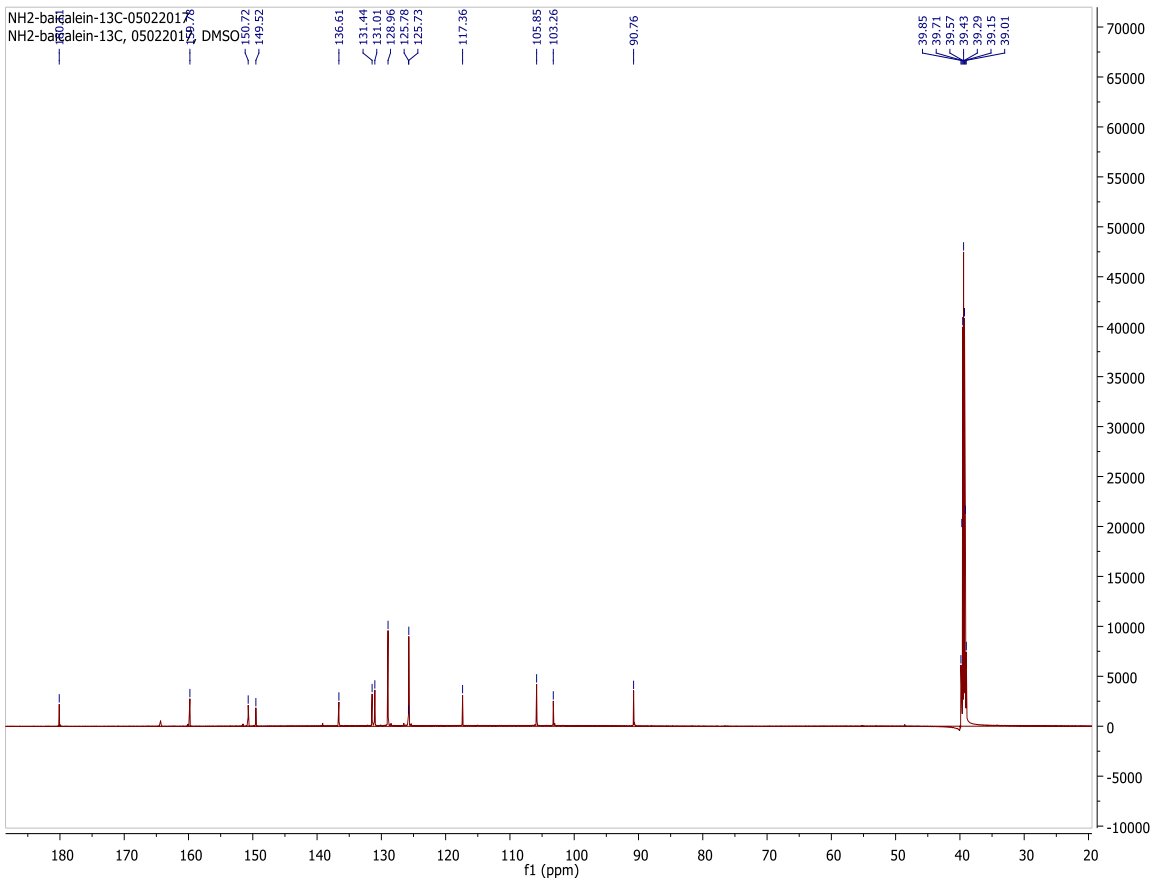

Figure S16. ^{13}C-NMR spectra of 5, 6-di-NH_2-baicalein (3) in d_6-DMSO.

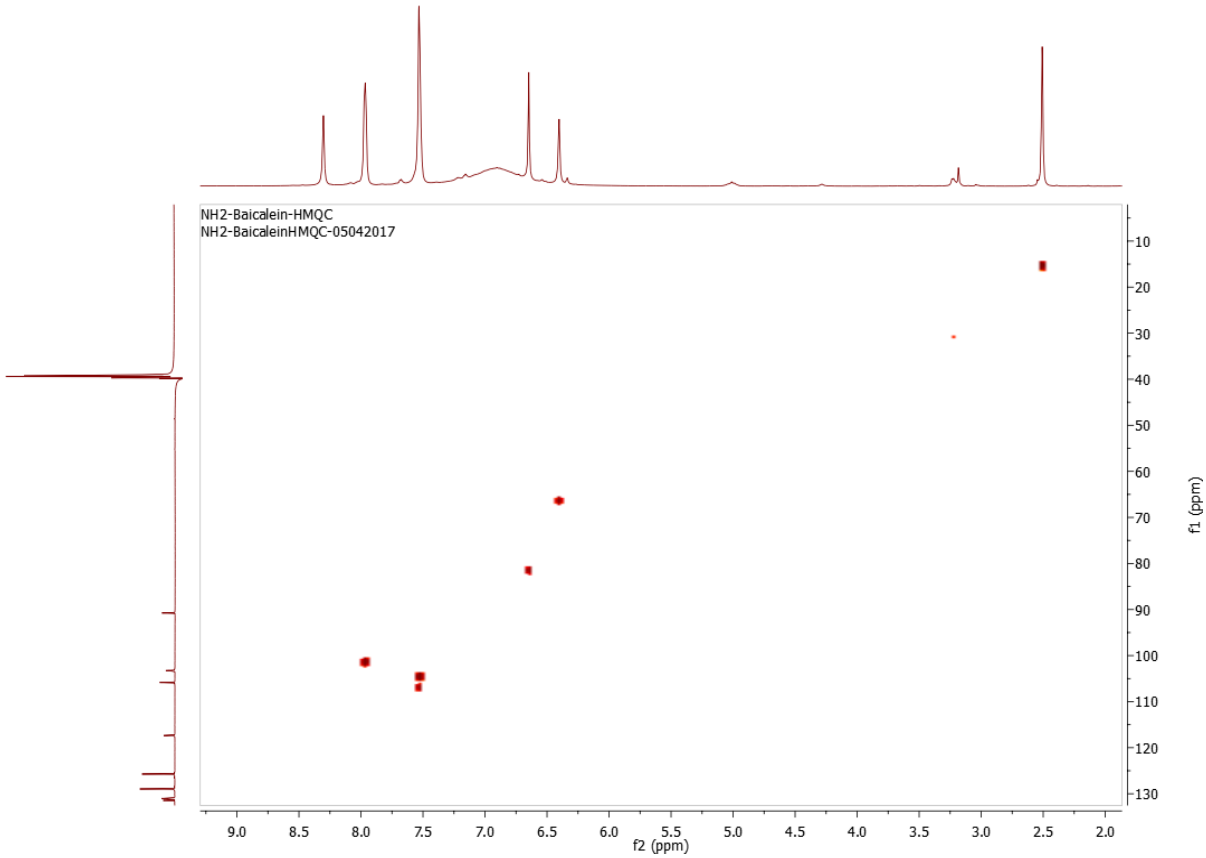

Figure S17. HMQC spectra of 5, 6-di-NH_2-baicalein (3) in d6-DMSO.

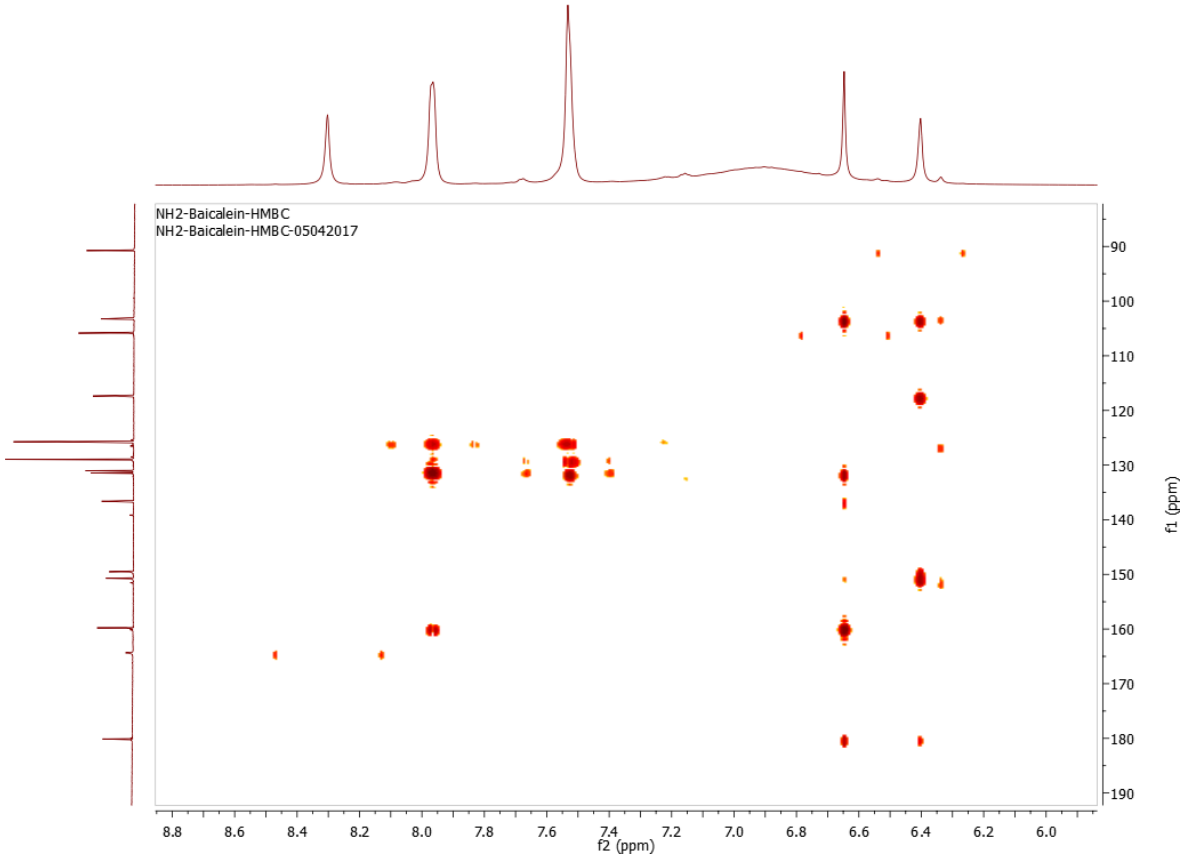

Figure S18. HMBC spectra of 5, 6-di-NH_2-baicalein (3) in d6-DMSO.

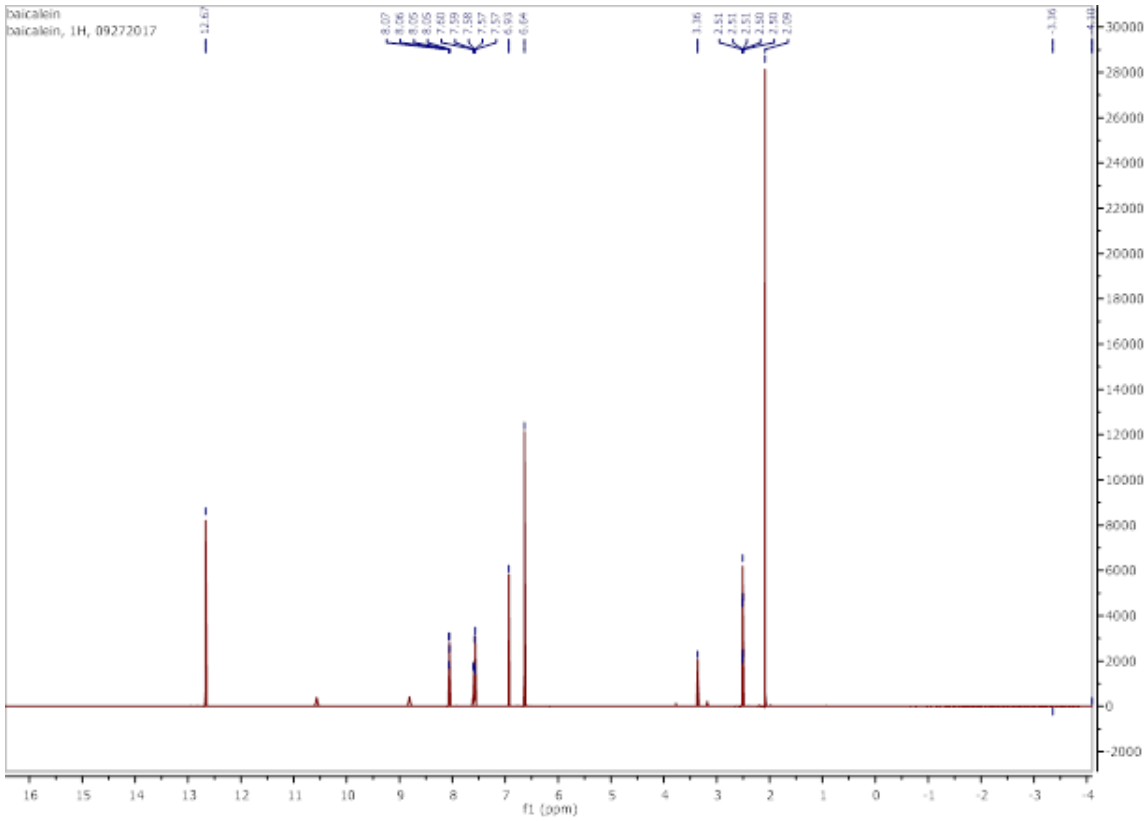

Figure S19. ^{1}H-NMR spectrum of baicalein in d6-DMSO.

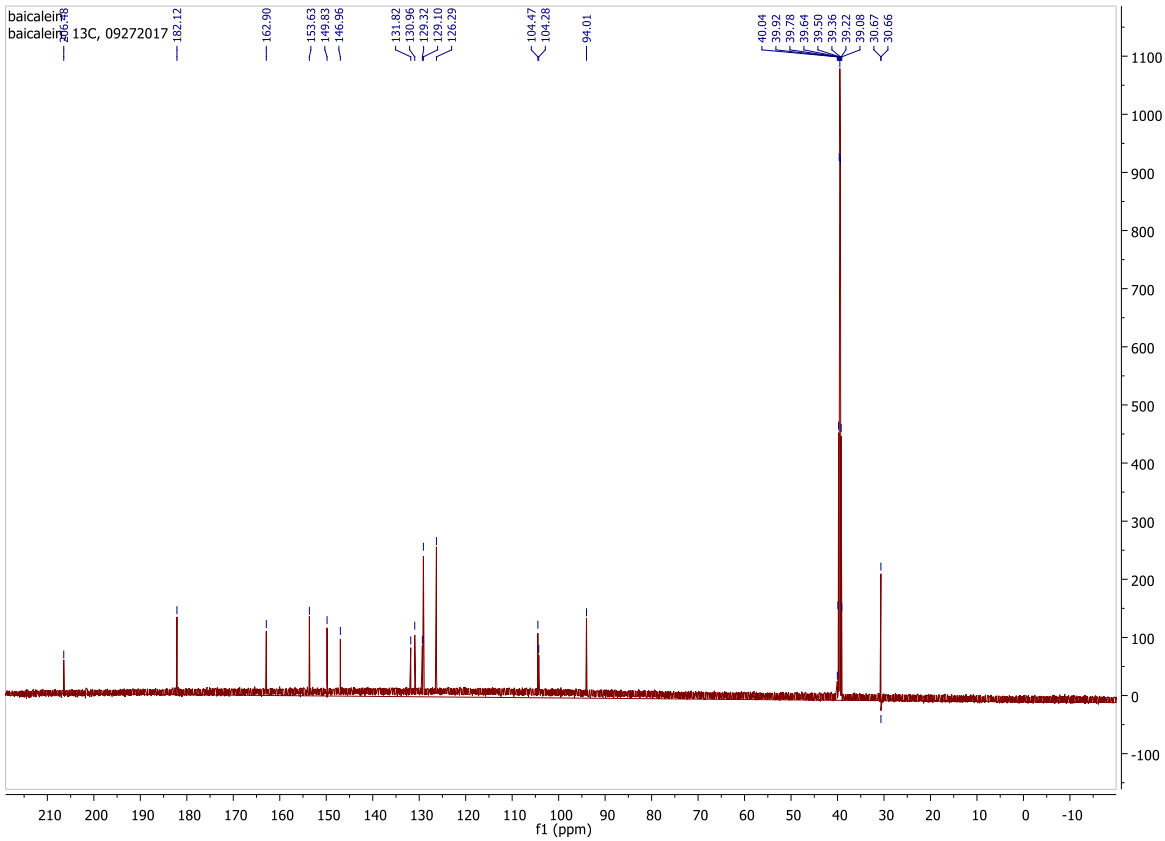

Figure S20. ^{13}C-NMR spectrum of baicalein in d6-DMSO.