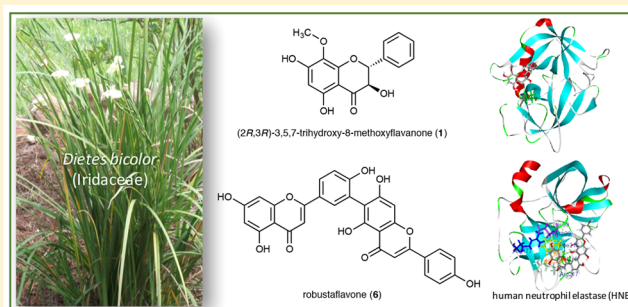


Probing the Antiallergic and Anti-inflammatory Activity of Biflavonoids and Dihydroflavonols from *Dietes bicolor*Irinay M. Ayoub,^{†,‡} Michal Korinek,^{‡,§,⊥,¶} Tsong-Long Hwang,^{||,▽} Bing-Hung Chen,^{§,△,□} Fang-Rong Chang,^{‡,⊥,δ} Mohamed El-Shazly,^{*,†,||,Ⓜ} and Abdel Nasser B. Singab^{*,†}[†]Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University, African Union Organization Street, Cairo 11566, Egypt[‡]Graduate Institute of Natural Products, College of Pharmacy, [§]Department of Biotechnology, College of Life Science, [⊥]Research Center for Natural Products & Drug Development, and ^δCenter for Infectious Disease and Cancer Research, Kaohsiung Medical University, Kaohsiung 80708, Taiwan^{||}Graduate Institute of Natural Products, College of Medicine, and Chinese Herbal Medicine Research Team, Healthy Aging Research Center, Chang Gung University, Taoyuan 33302, Taiwan[▽]Research Center for Chinese Herbal Medicine, Research Center for Food and Cosmetic Safety, and Graduate Institute of Health Industry Technology, College of Human Ecology, Chang Gung University of Science and Technology, Taoyuan 33302, Taiwan[△]Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung 80708, Taiwan[□]The Institute of Biomedical Sciences, National Sun Yat-sen University, Kaohsiung 804, Taiwan[¶]Department of Pharmaceutical Biology, Faculty of Pharmacy and Biotechnology, German University in Cairo, Cairo 11432, Egypt

S Supporting Information

ABSTRACT: *Dietes bicolor* (Iridaceae) is an ornamental plant used by African local healers to treat diarrhea and dysentery. A new dihydroflavonol, (2R,3R)-3,5,7-trihydroxy-8-methoxyflavanone (1); two known dihydroflavonols, *trans*-3-hydroxy-5-methoxy-6,7-methylenedioxyflavanone (2) and *trans*-3-hydroxy-5,7-dimethoxyflavanone (3); the known isoflavone orobol 7,3'-di-O-methyl ether (4); the known biflavones lanaroflavone (5), robustaflavone (6), and amentoflavone (7); and β -sitosterol (8) were isolated from the CH₂Cl₂ fraction of *D. bicolor* leaves. The extract showed potent activity in antiallergic and anti-inflammatory assays. The structures of the isolates were identified by spectroscopic and spectrometric methods. Compounds 6 and 7 (400 μ M) exhibited antiallergic activity by inhibiting antigen-induced β -hexosaminidase release at 45.7% and 46.3%, respectively. Moreover, 6 and 7 exerted anti-inflammatory activity as demonstrated by the inhibition of superoxide anion generation with an IC₅₀ value of 1.0 μ M as well as the inhibition of elastase release with IC₅₀ values of 0.45 and 0.75 μ M, respectively. The anti-inflammatory activity was further explained by the virtual docking of the isolated compounds to the binding sites in the human neutrophil elastase (HNE) crystal structure using Discovery Studio 2.5. It was concluded that the biflavonoids bind directly to HNE and inhibit its enzymatic activity based on the CDocker algorithm. The data provided evidence for the potential use of *D. bicolor* against certain diseases related to allergy and inflammation.



Incidences of allergic diseases such as seasonal rhinitis, food allergy, and asthma have increased over the last few decades. Such pathological reactions affect approximately 20% of the world population.¹ A complex network of specialized cells and cellular mediators controls allergic reactions. Among the most important cells affecting allergic reactions are mast cells. They serve as important effector cells that play a crucial role in both immediate and late-phase type I allergic reactions. The mast cells activated by allergen-induced cross-linking of IgE bound by the high-affinity IgE receptors (Fc ϵ RI) trigger degranulation, leading to the release of various mediators from granules, including β -hexosaminidase, histamine, and serotonin, that ultimately contribute to acute and chronic allergic reactions.^{2,3} The suppression of mast-cell-mediated inflammatory responses

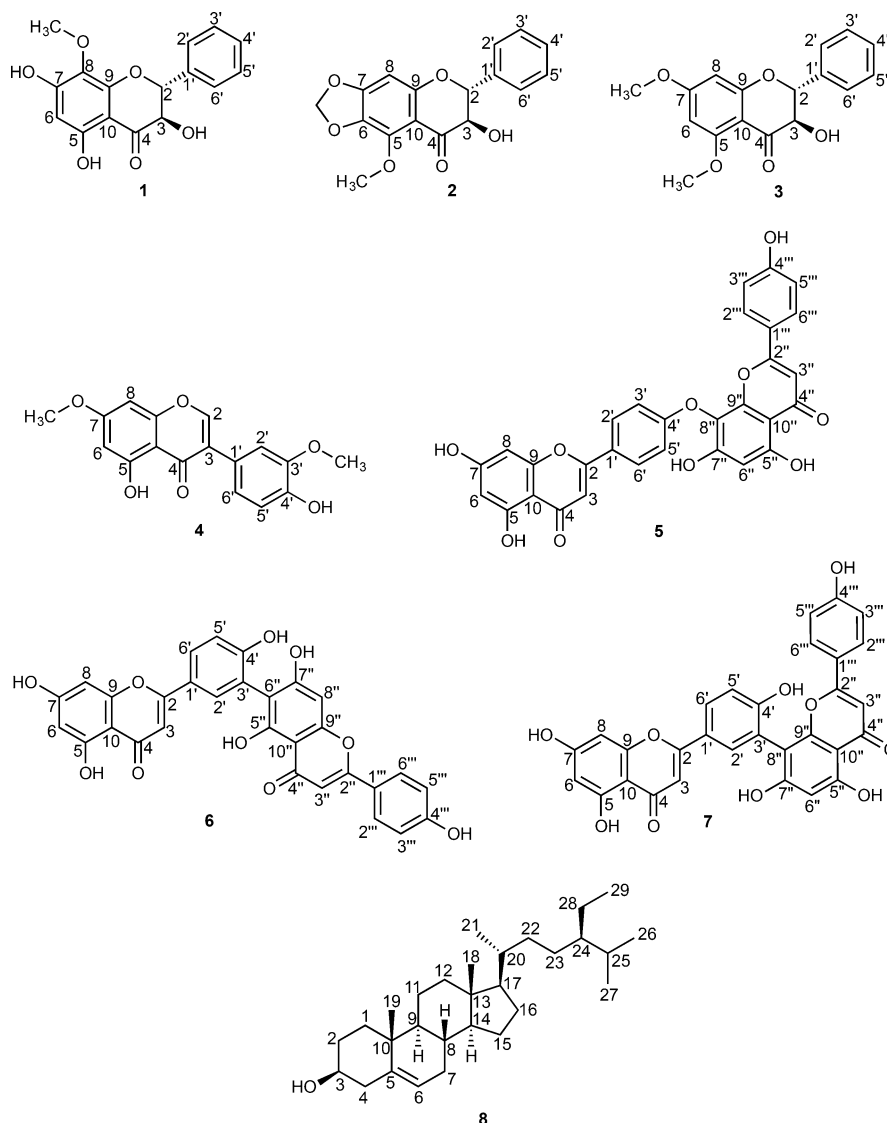
is particularly important to avoid immediate hypersensitivity reactions in atopic individuals. RBL-2H3, a rat basophilic leukemia cell line,⁴ is utilized to study mast cell activation and to screen compounds with antiallergic potential.^{5–7} The release of β -hexosaminidase is regarded as a useful indicator to evaluate allergic and inflammatory responses in mast cells.⁶

Neutrophils are important in the innate immune system, serving as a defensive wall against invading pathogens and are often the first cells to be recruited to the inflammatory sites. Upon exaggerated activation of neutrophils, the cells undergo

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Chart 1



pathological degranulation and respiratory burst, resulting in tissue damage, which is a common feature of many inflammatory diseases.⁸ Previous studies indicated that the level of elastase and the production of superoxide, a precursor of other reactive oxygen species, serve as important markers of inflammation in human neutrophils.⁹

In the last century, several classes of therapeutic agents were developed to alleviate inflammation and allergy. The most common drugs used to treat inflammatory response are nonsteroidal anti-inflammatory drugs (NSAIDs). These drugs are effective in relieving symptoms of inflammation but are associated with serious side effects that restrict their usage.¹⁰ Allergic conditions were treated with several classes of antihistaminic drugs that demonstrated high potency in relieving unpleasant allergic symptoms but exhibited some adverse effects.¹¹ Nature continues to provide an arsenal of secondary metabolites with unique activity and superior safety profiles. A plethora of anti-inflammatory agents were discovered in the last few decades, but a limited number of compounds were found to possess antiallergic activity.^{12,13} Certain plant genera are still used by folk medicinal practitioners to treat inflammation and allergy without any scientific evidence

limiting their use and development as approved therapeutic agents.

Dietes is a small genus of rhizomatous plants composed of six species in the family Iridaceae. *Dietes bicolor* (Steud.) Sweet ex Klatt is an evergreen low herbaceous ornamental plant from Africa with sword-like leaves and prolific flowers and is widely used in commercial plantings.¹⁴ It is commonly known as yellow wild iris, peacock flower, or butterfly iris. The genus name "*Dietes*" is derived from the Greek "dis", which means twice, and "etes", which means an associate. It shares the characteristics of *Iris* and the African genus *Moraea*. The species name "*bicolor*" means two-colored.¹⁵

Despite the widespread use of *Dietes* sp. by local African healers, few studies reported their traditional use. Certain species of *Dietes* were used by traditional healers in South Africa for the treatment of diarrhea and dysentery either orally or as enemas. Infusion of the inner part of *D. iridioides* rhizomes was also used during childbirth and in the treatment of hypertension.¹⁶ A previous study proved the antihypertensive effect of *D. iridioides* leaves' aqueous extract, which showed 80% inhibition of angiotensin converting enzyme (ACE).¹⁷ In our ongoing research of this unexplored South African plant, we

reported the antimicrobial activity of the crude alcoholic extracts of the leaves, flowers, and rhizomes of *D. bicolor*. Interestingly, no cytotoxic activity was observed in mammalian cells.¹⁸ We also studied the volatile oil composition of *D. bicolor* as well as the antimicrobial activity of the essential oils of the leaves and flowers. The essential oil of the leaves showed more potent antimicrobial activity as compared with the oil of the flowers against most of the assessed bacteria and fungi.¹⁹ A literature search indicated the absence of any report dealing with the phytochemical composition and biological activities of *D. bicolor*.

The purpose of the current study was to evaluate the antiallergic and anti-inflammatory activities of *D. bicolor* extracts, fractions, and isolated compounds. Phytochemical investigation of the biologically active fraction was carried out to isolate and identify its major secondary metabolites that might be responsible for such activities. One new dihydroflavonol (**1**), two known dihydroflavonols (**2** and **3**), isoflavone (**4**), three biflavones (**5**–**7**), and steroid (**8**) were isolated from a CH₂Cl₂ extract of the leaves of *D. bicolor*. The isolated compounds were subjected to in vitro antiallergic and anti-inflammatory assays as well as an in silico molecular docking experiment to study their binding sites in a human neutrophil elastase (HNE) crystal structure using the CDocker algorithm.

RESULTS AND DISCUSSION

Compound **1** was obtained as a light yellow, amorphous powder. The HRESIMS data of compound **1** showed a protonated molecule at m/z 303.0860 [$M + H^+$] (calcd 303.0863) in the positive ion mode, indicating a molecular weight of 302 and a molecular formula of C₁₆H₁₄O₆ (10 indices of hydrogen deficiency), which was supported by ¹³C NMR data. The IR spectrum showed the presence of a hydroxy (3385 cm⁻¹), carbonyl (1722 cm⁻¹), and aromatic (1582 cm⁻¹) functionalities. The UV spectrum of **1** exhibited an absorption maximum at 294 nm, suggesting a dihydroflavonol.²⁰ Dihydroflavonols generally exhibit an intense band II with a shoulder or a low-intensity band I, due to the absence of conjugation between the A- and B-rings.²¹ The ¹³C NMR data indicated 16 carbons in the structure of **1**. The ¹H, ¹³C, and HSQC NMR data of **1** (Table 1) revealed the presence of a methoxy (δ_H 3.92, s; δ_C 60.4, MeO-8), two methines (δ_H 5.46, d, J = 11.2; δ_C 84.6, CH-2; 4.97, d, J = 11.2; δ_C 73.4, CH-3), six aromatic methines (δ_H 6.45, s; δ_C 96.4, CH-6; δ_H 7.80–7.82, m, δ_C 128.6, CH-2', 6'; δ_H 7.42–7.46, m, δ_C 128.7, CH-3', 5'; δ_H 7.37–7.39, m; δ_C 129.0, CH-4'), six additional aromatic carbons (δ_C 158.9, C-5; δ_C 161.7, C-7; δ_C 130.8, C-8; δ_C 156.3, C-9; δ_C 102.0, C-10; δ_C 138.5, C-1'), and a carbonyl carbon (δ_C 198.9, C-4). In the ¹H NMR spectrum, signals at δ_H 5.46 (H-2) and δ_H 4.97 (H-3) indicated a 3-substituted flavanone type and the deshielded shift of H-3 suggested an oxygen-bearing atom at C-3. The 3-hydroxy-substituted flavanone nucleus was further established by ¹³C NMR signals of C-2 (δ_C 84.6), C-3 (δ_C 73.4), and C-4 (δ_C 198.9) together with the COSY cross-peak between H-2 and H-3, as well as the HMBC correlations between H-3 (δ_H 4.97, d, J = 11.2 Hz) and C-2, C-1', and C-4 (Figure 1). The magnitude of the coupling constant ($J_{2,3}$ = 11.2 Hz, in pyridine-*d*₅ or 12.0 Hz, in CDCl₃) indicated a *trans*-type dihydroflavonol,²² implying that H-2 and H-3 are *trans*-diaxial, whereas the B-ring and 3-hydroxy group are *trans*-diequatorially oriented.²³ Three multiplets of five protons at δ_H 7.37, 7.42, and 7.80 indicated the presence of an

Table 1. ¹H NMR and ¹³C NMR Data of Compound **1** in Pyridine-*d*₅^a

position	1 δ_H , mult, J in Hz	1 δ_C
2	5.46, d (11.2)	84.6, CH
3	4.97, d (11.2)	73.4, CH
4		198.9, C
5		158.9, C
6	6.45, s	96.4, CH
7		161.7, C
8		130.8, C
9		156.3, C
10		102.0, C
1'		138.5, C
2'	7.80–7.82, m	128.6, CH
3'	7.42–7.46, m	128.7, CH
4'	7.37–7.39, m	129.0, CH
5'	7.42–7.46, m	128.7, CH
6'	7.80–7.82, m	128.6, CH
MeO-8	3.92, s	60.4, CH ₃

^a¹H NMR spectra were measured at 400 MHz and ¹³C NMR spectra at 100 MHz in pyridine-*d*₅; chemical shifts are in ppm.

unsubstituted B-ring, which was further confirmed by NOESY correlations (Figure 1). The spectroscopic data suggested that **1** is a flavanone containing a methoxy (δ_H 3.92, 3H, s, MeO-8; δ_C 60.4, MeO-8) and three hydroxy groups. The downfield shift of the methoxy carbon signal at 60.4 ppm indicated its position to be either at C-8 or at C-6, being di-*ortho*-substituted by two oxygen functionalities.²⁴ The interpretation of the ¹³C NMR data suggested an 8-methoxyflavanone rather than a 6-methoxyflavanone.^{24–26} The HMBC spectrum showed correlations between the aromatic proton singlet at δ_H 6.45 and five carbons at δ_C 102.0 (C-10), 130.8 (C-8), 161.7 (C-7), 158.9 (C-5), and 198.9 (C-4); however, no correlation was observed with C-9 (δ_C 156.3). Therefore the signal δ_H 6.45 was assigned to H-6. Furthermore, HMBC correlations were observed between the methoxy proton signal at δ_H 3.92 and the C-8 oxyaryl signal at δ_C 130.8, indicating the position of the methoxy group at C-8 as shown in the structure of **1**. Additionally, the UV spectrum of **1**, after the addition of AlCl₃, showed a bathochromic shift of 23 nm in band II, which further confirmed the position of the methoxy group at C-8 rather than at C-6. The presence of a methoxy substituent at C-6 would result in a smaller bathochromic shift due to steric hindrance of the complex with HO-5.²⁷ The fragment ion peaks observed in the ESIMS and ESIMS² spectra of **1** (m/z 300.9 [$M - 1$]⁺, m/z 255.8 [$M - H_2O - CO$]⁺, m/z 214.1 [$C_{13}H_{10}O_3$]⁺, m/z 180.6 [$C_8H_5O_5$]⁺, and m/z 166.5 [⁰3A]⁺) confirmed the proposed structure (Figures S10 and S11, Supporting Information) and were in agreement with the established fragmentation pattern of dihydroflavonols.²⁸ The absolute configuration of **1** was deduced by means of the electronic circular dichroism (ECD) spectrum. Negative and positive Cotton effects were observed in the π – π^* and n – π transition regions, respectively, suggesting a (2*R*,3*R*) configuration.²³ Therefore, **1** was identified as the new (2*R*,3*R*)-3,5,7-trihydroxy-8-methoxyflavanone. It is noteworthy to mention that tetrasubstituted and fully oxygenated A-ring flavonoids with an unsubstituted B-ring are rare in nature.²⁶

The ¹H NMR spectrum of **2** suggested that it possessed a dihydroflavonol skeleton with an unsubstituted B-ring.^{29–31} Compound **2** was identified as *trans*-3-hydroxy-5-methoxy-6,7-

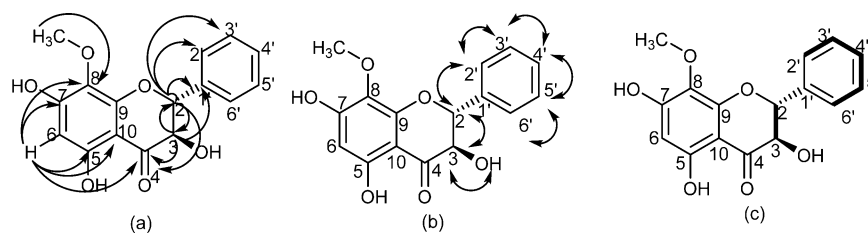


Figure 1. Important HMBC correlations (a), NOESY correlations (b), and COSY correlations (c) of compound 1.

Table 2. Inhibitory Activity of *D. bicolor* Extracts and Fractions on A23187-Induced and Antigen-Induced β -Hexosaminidase Release Degranulation in Mast Cells

sample	viability, RBL-2H3 ^a	inhibition of A23187-induced β -hexosaminidase release ^b			inhibition of antigen-induced β -hexosaminidase release ^b		
	100 μ g/mL	10 μ g/mL	100 μ g/mL	IC ₅₀ (μ g/mL) ^c	10 μ g/mL	100 μ g/mL	IC ₅₀ (μ g/mL) ^c
DBL	89.5 $\pm$ 3.5%	10.0 $\pm$ 14.1% ^d	0.0 $\pm$ 0.0% ^d	—	N/A	N/A	N/A
DBL-H	95.0 $\pm$ 12.7%	4.3 $\pm$ 4.5%	97.7 $\pm$ 4.0%**	54.0	0.0 $\pm$ 0.0%	94.7 $\pm$ 8.4%**	57.5
DBL-DCM	94.5 $\pm$ 0.7%	10.3 $\pm$ 3.1%*	98.3 $\pm$ 2.9%**	50.6	3.0 $\pm$ 5.2%	95.7 $\pm$ 7.5%**	55.6
DBL-But	95.0 $\pm$ 2.8%	3.5 $\pm$ 2.1% ^d	1.0 $\pm$ 1.4% ^d	—	N/A	N/A	N/A
DBR	91.0 $\pm$ 0.0%	enhancing ^{d,e}	enhancing ^{d,e}	—	N/A	N/A	N/A
DBF	80.5 $\pm$ 9.2%	3.5 $\pm$ 4.9% ^d	13.0 $\pm$ 1.4% ^d	—	N/A	N/A	N/A

^aResults are presented as percentage of viability compared with untreated control (MTT assay); mean $\pm$ SD ($n = 2$). ^bDexamethasone (50 nM) inhibited 83.0 $\pm$ 4.5% of A23187-induced β -hexosaminidase release and 69.5 $\pm$ 4.9% of antigen-induced β -hexosaminidase release; results are presented as mean $\pm$ SD ($n = 3$); * $P < 0.05$, ** $P < 0.001$ compared with the control value (A23187 or antigen). ^cIC₅₀ values express the concentration of the sample required to inhibit degranulation by 50%. ^dResults are presented as mean $\pm$ SD ($n = 2$). ^eThe samples showed enhancing effects on β -hexosaminidase release in the presence of A23187. DBR 109.0 $\pm$ 9.9% at 10 μ g/mL and 213.0 $\pm$ 26.9% at 100 μ g/mL. Compared with A23187 (as 100%). N/A, not applicable. DBL, *D. bicolor* leaves crude MeOH extract; DBL-H, *D. bicolor* leaves *n*-hexane fraction; DBL-DCM, *D. bicolor* leaves CH₂Cl₂ fraction; DBL-But, *D. bicolor* leaves *n*-BuOH fraction; DBR, *D. bicolor* roots crude MeOH extract; DBF, *D. bicolor* flowers crude MeOH extract.

methylenedioxyflavanone, which was confirmed by DEPT, HSQC, HMBC, and NOESY experiments. This is the first report of this compound in Iridaceae and the fourth report in nature.

The similarity of the ¹H NMR data of 3 compared to compounds 1 and 2 indicated that 3 is also a dihydroflavonol derivative with an unsubstituted B-ring. The ¹H NMR assignments were in good agreement with the data of a 3-hydroxyflavanone.^{32,33} Compound 3 was identified as *trans*-3-hydroxy-5,7-dimethoxyflavanone. This is the first report of 3 in the genus, the second report in Iridaceae, being recently isolated from *Iris pseudacorus*,³³ and the third report in nature.³⁴

The molecular formula of 4 was established as C₁₇H₁₄O₆ by ESIMS m/z 314.98 [M + H]⁺ and the ¹³C NMR data. By comparing its spectroscopic data with reported data,^{35,36} compound 4 was identified as 5,4'-dihydroxy-7,3'-dimethoxyisoflavone, known as orobol 7,3'-di-*O*-methyl ether.

The structure of 5 was elucidated as lanaroflavone, a biflavonoid showing a C-4'-*O*-C-8'' ether linkage. In contrast to most other biflavonoids, its interflavonyl link is not a C—C bond but a diaryl ether link. Spectroscopic data of 5 were in good agreement with published data.^{37,38} This is the first report of this compound in the Iridaceae. The ¹H NMR spectrum of 6 showed two distinct sets of flavonoid resonances indicating a biflavonoid. Spectroscopic data were consistent with those published for the C-3'—C-6'' linked biflavone, robustaflavone.^{39–41} The proposed structure was confirmed by ESIMS, HSQC, HMBC, COSY, and NOESY experiments. Consequently, 6 was identified as robustaflavone. This is the first report of robustaflavone in the Iridaceae. The ¹H NMR spectrum of 7 showed two distinct sets of flavonoid resonances indicating a biflavonoid, as evidenced by the mass spectrum (m/z 539.22 [M + H]⁺). Spectroscopic data were in agreement

with the literature values of the C-3'—C-8'' linked biflavone, amentoflavone.^{40,42,43} The proposed structure was further confirmed by ESIMS, HSQC, HMBC, COSY, and NOESY experiments. Thus, compound 7 was identified as amentoflavone. The ¹H NMR spectroscopic data of compound 8 were consistent with the reported data of β -sitosterol.⁴⁴

Bioactivity of *D. bicolor*. The extracts and fractions were evaluated for their potential antiallergic and anti-inflammatory activities. First, the extracts and fractions were evaluated for their degranulation inhibitory effect in mast cells. Based on the MTT viability assay results, all *D. bicolor* extracts and fractions were considered nontoxic to the RBL-2H3 cell line at a maximum concentration of 100 μ g/mL (Table 2). Samples were subjected to an A23187-induced degranulation assay evaluating the release of β -hexosaminidase from RBL-2H3 cells, and the most potent samples were further tested in antigen-induced (anti-DNP IgE plus DNP-BSA) β -hexosaminidase assay. The pretreatment with *n*-hexane (DBL-H) and the CH₂Cl₂ (DBL-DCM) fractions significantly suppressed A23187-induced degranulation in RBL-2H3 cells with IC₅₀ values of 54.0 and 50.6 μ g/mL, respectively (Table 2). The potent antiallergic activity of the *n*-hexane (DBL-H) and CH₂Cl₂ (DBL-DCM) fractions was further confirmed by an antigen-mediated β -hexosaminidase release degranulation assay, exhibiting IC₅₀ values of 57.5 and 55.6 μ g/mL, respectively (Table 2). Such antiallergic activity supports previous reports on the antiasthmatic activity of some Iridaceae plants.⁴⁵ Interestingly, *D. bicolor* rhizome extract (DBR) showed pro-allergic (213.0 $\pm$ 26.9% release of β -hexosaminidase compared to 100% release of control, Table 2) as well as pro-inflammatory effects (46.5 $\pm$ 4.6% release of elastase, Table 3). Allergic properties such as skin irritations were reported for certain roots belonging to Iridaceae.⁴⁶

Table 3. Effect of *D. bicolor* on Superoxide Anion Generation and Elastase Release in fMLF/CB-Induced Human Neutrophils

sample	superoxide anion generation ^a		elastase release ^a	
	IC ₅₀ (μg/mL) ^b	inhibition% at 10 μg/mL	IC ₅₀ (μg/mL) ^b	inhibition% at 10 μg/mL
DBL	>10	44.2 ± 4.0**	7.6 ± 1.3	61.3 ± 4.4**
DBL-H	>10	46.5 ± 2.8**	5.2 ± 0.4	63.7 ± 4.8**
DBL-DCM	2.5 ± 0.4	94.7 ± 2.4**	2.6 ± 0.7	116.1 ± 3.0**
DBL-But	6.0 ± 1.8	56.5 ± 3.0**	7.2 ± 1.4	60.8 ± 5.6**
DBL-aq	>10	33.9 ± 6.4*	>10	17.9 ± 6.5
DBR	>10	27.1 ± 2.1**	enhancing ^c	enhancing ^c
DBF	>10	46.6 ± 7.0*	>10	45.9 ± 3.1**

^aIC₅₀ values and percentage of inhibition at 10 μg/mL concentration. Results are presented as mean ± SEM ($n = 3-4$). * $P < 0.05$, ** $P < 0.001$ compared with the control value (formyl-methionyl-leucyl-phenylalanine/cytochalasin B, fMLF/CB). ^bConcentration necessary for 50% inhibition (IC₅₀). ^cThe sample showed enhancing effects on elastase release at 10 μg/mL (46.5 ± 4.6%) in the presence of CB. Compared with fMLF/CB (as 100%). DBL, *D. bicolor* leaves crude MeOH extract; DBL-H, *D. bicolor* leaves *n*-hexane fraction; DBL-DCM, *D. bicolor* leaves CH₂Cl₂ fraction; DBL-But, *D. bicolor* leaves *n*-BuOH fraction; DBL-aq, *D. bicolor* leaves MeOH(aq) fraction; DBR, *D. bicolor* roots crude MeOH extract; DBF, *D. bicolor* flowers crude MeOH extract.

The anti-inflammatory activity of *D. bicolor* extracts and fractions was assessed by superoxide anion generation and elastase release assays in human neutrophils. *D. bicolor* leaf extract and its fractions showed potent anti-inflammatory activity (Table 3), with the CH₂Cl₂ fraction exerting potent inhibition of superoxide anion generation (IC₅₀ value of 2.5 μg/mL) and inhibition of elastase release in human neutrophils (IC₅₀ value of 2.6 μg/mL). Several plants from the Iridaceae family were previously reported to possess anti-inflammatory and immunomodulating properties;⁴⁷⁻⁵⁰ however, no report was found on the anti-inflammatory activity of *Dietes* species.

Bioactivity of the Isolated Compounds. The isolated compounds were tested for their antiallergic and anti-inflammatory activities. Orobol 7,3'-di-O-methyl ether (4) showed a toxic effect (31%) toward RBL-2H3 cells at 400 μM. Previous studies revealed that neither isoflavones (4) nor dihydroflavonols (1-3), in comparison with other flavonoids, were active in the antigen-induced degranulation assay in RBL-2H3 cells (IC₅₀ values >100 μM).⁵¹ The antiallergic activity of the isolated biflavonoids was assessed. The results showed that robustaflavone (6) and amentoflavone (7) exerted significant inhibition of A23187-induced β-hexosaminidase release (45.3 ± 7.6% and 42.3 ± 3.8% at 400 μM, respectively) and antigen-induced β-hexosaminidase release (49.0 ± 9.2% and 50.0 ± 9.2% at 400 μM, respectively) in RBL-2H3 cells (Table 4). Amentoflavone was previously reported to inhibit A23187-induced histamine release from peritoneal rat mast cells.⁵² Therefore, the antiallergic activity of *D. bicolor* may be partially attributed to the presence of biflavonoids.

Evaluation of the anti-inflammatory activity revealed that orobol 7,3'-di-O-methyl ether (4) significantly inhibited superoxide anion generation (48.9 ± 4.8%) at 10 μM (Table 5). Isoflavones were previously reported to inhibit nitric oxide (NO), cytokines, TNF-α, and leukotriene B₄ (LTB₄) production and suppress iNOS, cyclooxygenase-2 (COX-2)

Table 4. Inhibitory Activity of Pure Compounds Isolated from *D. bicolor* on A23187-Induced and Antigen-Induced β-Hexosaminidase Release in Mast Cells

sample	viability, RBL-2H3 ^a		inhibition of A23187-induced β -hexosaminidase release, RBL-2H3 cells ^b					inhibition of antigen-induced β -hexosaminidase release, RBL-2H3 cells ^b				
	400 μ M		1 μ M	10 μ M	100 μ M	200 μ M	400 μ M	1 μ M	10 μ M	100 μ M	200 μ M	400 μ M
orobol 7,3'-di-O-methyl ether (4)	69.0 $\pm$ 3.6%		0.3 $\pm$ 0.6%	1.7 $\pm$ 2.9%	23.7 $\pm$ 1.5%**	22.7 $\pm$ 6.8%**	TOXIC ^c	2.3 $\pm$ 2.1%	1.3 $\pm$ 1.2%	9.0 $\pm$ 4.4%	18.0 $\pm$ 8.7% *	TOXIC ^c
robustaflavone (6)	92.3 $\pm$ 7.5%		2.3 $\pm$ 4.0%	5.3 $\pm$ 9.2%	6.0 $\pm$ 10.4%	2.7 $\pm$ 4.6%	45.3 $\pm$ 7.6%**	4.0 $\pm$ 6.9%	3.0 $\pm$ 5.2%	2.7 $\pm$ 4.6%	3.0 $\pm$ 5.2%	49.0 $\pm$ 9.2%**
amentoflavone (7)	97.7 $\pm$ 4.0%		2.0 $\pm$ 3.5%	6.7 $\pm$ 4.2%	0.0 $\pm$ 0.0%	3.3 $\pm$ 2.9%	42.3 $\pm$ 3.8%**	4.7 $\pm$ 7.2%	1.3 $\pm$ 1.5%	2.7 $\pm$ 4.6%	7.7 $\pm$ 6.8%	50.0 $\pm$ 9.2%**

^aResults are presented as percentage of viability at 400 μ M compared with untreated control (MTT assay); mean $\pm$ SD (n = 3). ^bDexamethasone (10 nM) inhibited 80.7 $\pm$ 3.8% of A23187-induced β -hexosaminidase release and 79.7 $\pm$ 2.5% of antigen-induced β -hexosaminidase release; results are presented as mean $\pm$ SD (n = 3); * p < 0.05, ** p < 0.001 compared with the control value (A23187 or antigen) “TOXIC” indicates that the sample exerted cytotoxic effects toward RBL-2H3 cells (viability less than 80%).

^aResults are presented as percentage of viability at 400 μM compared with untreated control (MTT assay); mean ± SD ($n = 3$). ^bDexamethasone (10 nM) inhibited 80.7 ± 3.8% of A23187-induced β-hexosaminidase release and 79.7 ± 2.5% of antigen-induced β-hexosaminidase release; results are presented as mean ± SD ($n = 3$). * $P < 0.05$, ** $P < 0.001$ compared with the control value (A23187 or antigen). ^c"TOXIC" indicates that the sample exerted cytotoxic effects toward RBL-2H3 cells (viability less than 80%).

Table 5. Effect of the Isolated Compounds from *D. bicolor* on Superoxide Anion Generation and Elastase Release in fMLF/CB-Induced Human Neutrophils

sample	superoxide anion generation ^a		elastase release ^a	
	IC ₅₀ (μM) ^b	inhibition% at 10 μM	IC ₅₀ (μM) ^b	inhibition% at 10 μM
orobol 7,3'-di-O-methyl ether (4)	>10	48.9 ± 4.8**	>10	27.1 ± 3.2*
robustaflavone (6)	1.01 ± 0.17	105.1 ± 1.1**	0.45 ± 0.11	103.2 ± 0.6**
amentoflavone (7)	1.01 ± 0.14	104.1 ± 0.2**	0.75 ± 0.18	108.5 ± 4.2**
LY294002 ^c	1.25 ± 0.16	96.5 ± 3.0**	3.44 ± 0.35	71.5 ± 5.5**

^aIC₅₀ values and percentage of inhibition at 10 μM concentration. Results are presented as mean ± SEM (*n* = 3–4). **P* < 0.05, ***P* < 0.001 compared with the control value (formyl-methionyl-leucyl-phenylalanine/cytochalasin B, fMLF/CB). ^bConcentration necessary for 50% inhibition (IC₅₀). ^cLY294002, the PI3K inhibitor, was used as a positive control.

expression, and NF-κB activation.⁵³ Furthermore, the biflavonoids robustaflavone (6) and amentoflavone (7) significantly suppressed superoxide anion generation (IC₅₀ 1.01 and 1.01 μM, respectively) and inhibited elastase release (IC₅₀ 0.45 and 0.75 μM, respectively) in human neutrophils, exerting better activity in comparison with the positive control, the PI3K inhibitor LY294002 (IC₅₀ 1.25 μM in superoxide generation and 3.44 μM in elastase release assays) (Table 5). Interestingly, Xu et al. reported the inhibition of human neutrophil elastase enzymatic activity by robustaflavone (IC₅₀ 1.33 μM) and amentoflavone (IC₅₀ 1.27 μM), suggesting that the observed anti-inflammatory effect may be caused, to some extent, by the direct inhibition of elastase enzymatic activity.⁵⁴ The unique structural features and limited distribution of biflavonoids encouraged scientists to evaluate their biological activities including their anti-inflammatory effect. Amentoflavone suppressed NO and prostaglandin E₂ (PGE₂) levels, inhibited iNOS, cAMP-phosphodiesterase, and phospholipase A₂ (PLA₂) activity, and suppressed COX-2 expression via NF-κB inactivation and extracellular-signal-regulated kinase (ERK) inhibition.^{53,55–57} Interestingly, Yang et al. reported a marginal effect of robustaflavone on NO production and iNOS expression.⁵⁸ Our results add more weight to the reported anti-inflammatory effect of biflavonoids, targeting respiratory burst and degranulation in human neutrophils. Taken together, biflavonoids may play a significant role in the antiallergic and anti-inflammatory activities of *D. bicolor* leaves.

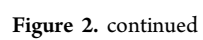
In Silico Molecular Modeling. To evaluate the possible interactions between the isolated compounds and HNE putative binding sites, molecular docking studies using the CDOCKER algorithm within Discovery Studio 2.5 were performed. To validate the enzyme structure model and the use of CDOCKER as a docking algorithm for HNE, the cocrystallized ligand GW475151 was redocked using the algorithm. The crystal structure of GW475151 extracted from PDB 1H1B was superimposed to the docked pose and agreed well with the conformation obtained, leading to an RMSD value between the calculated pose and the crystal structure of 1.4869. Docking studies revealed that the biflavonoids 5, 6, and 7 docked well into the HNE binding domain. The highest inhibition was exerted by lanaroflavone (5) followed by amentoflavone (7) and robustaflavone (6), as evidenced by their high fitting scores compared to the cocrystallized ligand, suggesting that these compounds should possess good binding affinities to HNE (Table 6). The catalytic activity is enhanced by a catalytic triad formed by three amino acid residues, His57, Asp102, and Ser195, that act as a general base that enhances the nucleophilicity of Ser195 forming a charge relay system through synchronous proton transfer from the hydroxy group of Ser195 to Asp102 through His57.⁵⁹ Lanaroflavone (5) formed a

Table 6. Free Binding Energies (Δ*G*) of the Isolated Compounds within the HNE Active Site Calculated in kcal/mol Using Discovery Studio 2.5

compound	binding energy Δ <i>G</i> (kcal/mol)	interaction amino acid residue
1	−14.8165	Val 216
2	4.16607	Val 216
3	−12.7993	Ser 195, Val 216
4	−19.5763	Ser 195, Phe215, Arg 217
5	−29.4279	His 57
6	−22.8225	Val 216, Phe 215, Arg 217, Asn 99, Arg 177
7	−23.4604	Ser 195, His 57
8	44.711	Ser 195
GW475151	−19.1622	Ser 195

hydrogen bond with His57 (Figure 2A). Amentoflavone (7) formed two hydrogen bonds with Ser195 and His57 (Figure 2B). Robustaflavone (6) formed three hydrogen bonds with Val216, Asn99, and Arg177, in addition to π–π interactions involving flavone A- and C-rings with Phe215 and Arg217 (Figure 2C). Orobol 7,3'-di-O-methyl ether (4) formed two hydrogen bonds with Ser195; moreover, π–π interactions were observed between the isoflavone C-ring with Phe215, in addition to π–σ interactions between the A-ring and Arg 217 (Figure 2D). The results were in good agreement with the experimental elastase enzymatic activity assay reported by Xu et al.⁵⁴ Furthermore, docking studies permitted the prediction of the bioactivity of the rare dihydroflavonols isolated from the DBL active fraction. Compounds 1 and 3 showed moderate inhibitory activity as revealed by the binding energies of −14.8165 and −12.7993 kcal/mol, respectively, compared with the lead compound cocrystallized with the human neutrophil elastase enzyme (−19.1622 kcal/mol). Compound 8 showed no inhibitory activity as evidenced by the endothermic binding energy (44.711 kcal/mol), suggesting unfavorable orientation for optimal binding.⁶⁰

In conclusion, we attempted to introduce *D. bicolor*, an important plant belonging to the Iridaceae family, to the phytotherapeutic arena. Despite its use by African local healers, no reports were traced regarding its phytochemistry and biological activities. Our findings revealed that the leaf fractions of *D. bicolor* possess antiallergic and anti-inflammatory activities. Phytochemical investigation of the bioactive CH₂Cl₂ fraction resulted in the isolation and identification of eight compounds, including biflavonoids and rare dihydroflavonols. The in silico molecular docking studies verified the anti-inflammatory activity, providing new insight into the potential use of biflavonoids as promising human neutrophil elastase inhibitory agents.



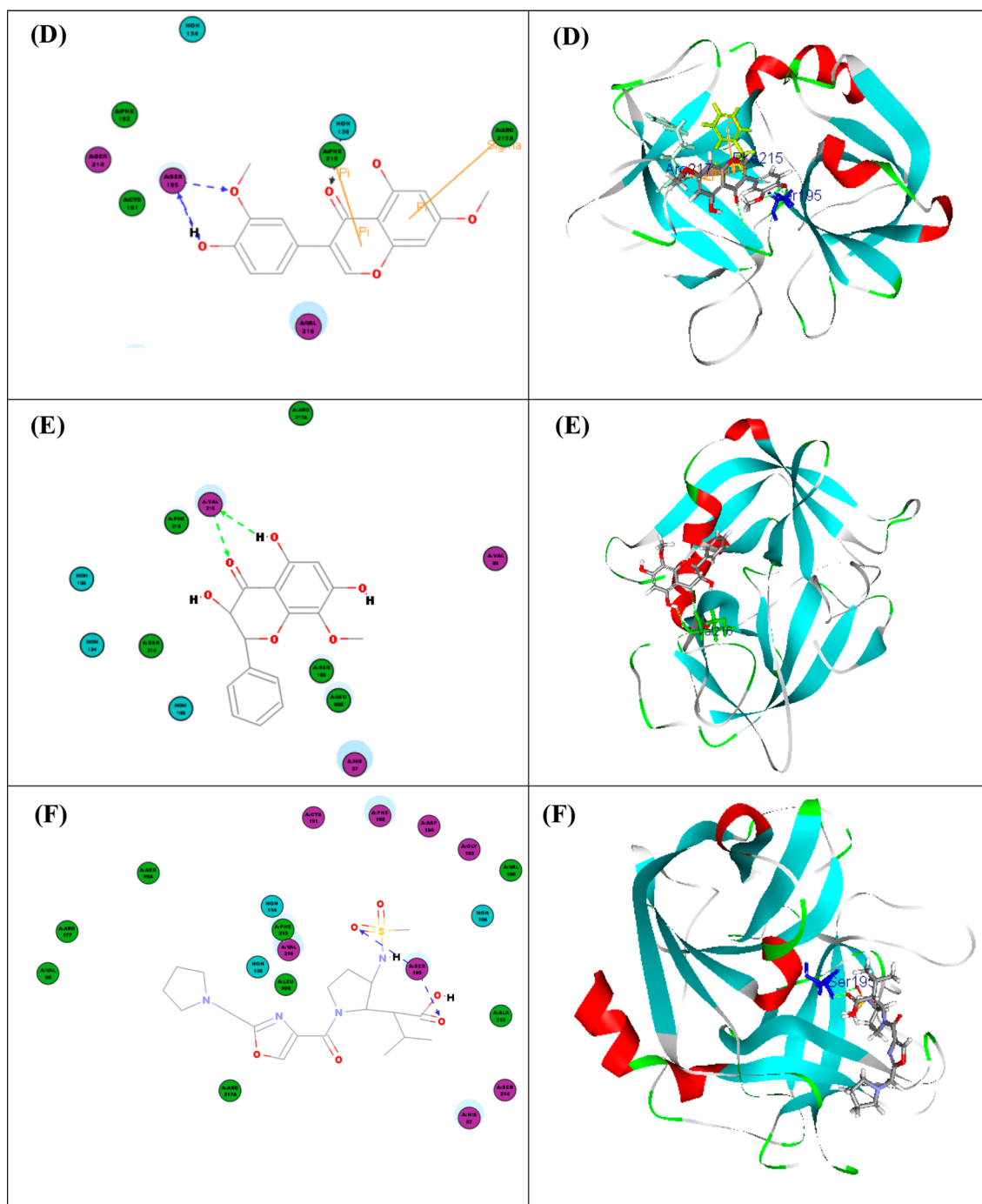


Figure 2. 2D and 3D ligand–enzyme interactions of high score compounds: lanaroflavone (5) (A); amentoflavone (7) (B); robustaflavone (6) (C); orobol 7,3'-di-O-methyl ether (4) (D); and (2*R*,3*R*)-3,5,7-trihydroxy-8-methoxyflavanone (1) (E) and GW475151 (F) with HNE (PDB ID: 1H1B). Residues are annotated with the three-letter amino acid code and their positions. Hydrophobic residues are colored green, whereas polar residues are colored purple. Water molecules are colored cyan. Hydrogen-bonding interactions are drawn with an arrowhead between the receptor and the ligand denoting the direction of the hydrogen bond. The arrow is drawn in green when the hydrogen bond is formed with the residue side chain. However, hydrogen bonds to the residue backbone are drawn in blue.

EXPERIMENTAL SECTION

General Experimental Procedures. A JASCO DIP-370 digital polarimeter and a JASCO P-2000 polarimeter (JASCO Inc., Tokyo, Japan) were used to measure optical rotations. UV spectra were obtained in MeOH using a JASCO V-530 spectrophotometer (JASCO Inc.). IR spectra were measured on a Hitachi 260-30 spectrophotometer (Hitachi Co., Tokyo, Japan). ECD spectra were recorded on a JASCO J-810 spectropolarimeter (JASCO Inc.). NMR spectra were obtained on a Unity Plus Varian NMR spectrometer (Varian, Palo

Alto, CA, USA); ^1H NMR (400 MHz, using CDCl_3 as a solvent for measurement), ^{13}C NMR (100 MHz), DEPT, HETCOR, COSY, NOESY, HMBC, and HRESIMS data were acquired on an FTHRMS-Orbitrap mass spectrometer (Thermo Finnigan). A JEOL JMS-SX/SX 102A mass spectrometer (JEOL Inc., Tokyo, Japan) was utilized to obtain LRFABMS and LREIMS data. Silica gel 60 (Merck, 230–400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F_{254} , 0.20 mm, and Merck, Kieselgel 60 F_{254} , 0.50 mm) were used for analytical and preparative TLC,

respectively. Spots were detected by spraying with 10% H_2SO_4 followed by heating on a hot plate.

Chemicals and Reagents. Dulbecco's modified Eagle's medium powder high glucose (DMEM), DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, streptomycin, LY294002, calcium ionophore A23187, dexamethasone, and *p*-nitrophenyl-*N*-acetyl- β -glucosaminide (*p*-NAG), as well as Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA), and dinitrophenyl-conjugated bovine serum albumin (DNP-BSA) from Pierce (Rockford, IL, USA). Mouse anti-DNP IgE (mIgE-DNP) antibodies were kindly provided by Dr. Daniel H. Conrad (Virginia Commonwealth University, Richmond, VA, USA). Column chromatography was done on silica gel 60 (Merck, Darmstadt, Germany). Precoated silica gel 60 F_{254} sheets on aluminum sheets (0.25 mm layer thickness; 20×20 cm, Merck, Darmstadt, Germany) were used for TLC. H_2SO_4 (10%) in MeOH (El-Nasr Co., Cairo, Egypt) was used as the spraying reagent. Dried plates were sprayed and heated at 105°C . Reagents for UV spectroscopic analysis were purchased from Sigma-Aldrich. Solvents used for extraction and isolation were of chromatographic purity, supplied by El-Nasr Co. Deuterated solvents were used for ^1H , ^{13}C , and 2D NMR spectroscopic measurements and were purchased from Sigma-Aldrich. Tetramethylsilane was used as an internal standard supplied by Sigma-Aldrich.

Plant Material. *D. bicolor* (Steud) Sweet ex Klatt was collected in January–April 2011 from a private botanical garden, Barageel, Giza, Egypt. The plant was authenticated by Mrs. Therese Labib, consultant of plant taxonomy at the Ministry of Agriculture and a former director of El-Orman Botanical Garden, Giza, Egypt, and Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC), Giza, Egypt. A voucher specimen (IA-10411) was deposited in the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Ain-Shams University.

Extraction and Isolation. Leaves of *D. bicolor* (2.7 kg) were air-dried, ground into a coarse powder, percolated in 80% MeOH (3×10 L), and filtered. The filtrate was evaporated in vacuo at 45°C until dryness and lyophilized to yield 341 g of the aqueous MeOH extract (DBL). The solid residue (300 g) was dissolved in $\text{H}_2\text{O}/\text{MeOH}$ (1:4) and successively partitioned with *n*-hexane, CH_2Cl_2 , and *n*-BuOH to give DBL-H (13.59 g), DBL-DCM (20.09 g), and DBL-But (48.96 g), respectively, and the remaining aqueous extract DBL-aq (195.5 g). The ground, air-dried rhizomes of *D. bicolor* (100 g) were percolated in distilled MeOH (3×500 mL) and filtered. The filtrate was evaporated in vacuo at low temperature (45°C) until dryness and lyophilized to obtain 11 g of the dried lyophilized *D. bicolor* total MeOH extract of the rhizomes (DBR). Fresh flowers of *D. bicolor* (100 g) were percolated in 80% MeOH (3×500 mL) and filtered. The filtrate was evaporated in vacuo at 45°C until dryness and lyophilized to give 8 g of *D. bicolor* flower aqueous MeOH extract (DBF). The CH_2Cl_2 fraction of *D. bicolor* leaves (DBL-DCM) showed potent antiallergic and anti-inflammatory activities, and the fraction was further purified to isolate the active components that might be responsible for the observed biological effects.

DBL-DCM (15 g) was adsorbed on 5 g of silica gel and applied to the top of an open column (120×5 cm) packed with silica gel 60 (400 g, Merck). The column was eluted with *n*-hexane, then *n*-hexane/ CH_2Cl_2 , and the polarity of the solvent system increased to 100% CH_2Cl_2 , followed by a gradient mixture of $\text{CH}_2\text{Cl}_2/\text{MeOH}$ until 100% MeOH. A total of 350 fractions were collected (200 mL each) and analyzed with silica gel F_{254} TLC plates using *n*-hexane/EtOAc and $\text{CH}_2\text{Cl}_2/\text{MeOH}$ as the developing solvent systems. Compounds were visualized under UV light at the wavelength of 254 and 365 nm. Chromatograms were sprayed with 10% H_2SO_4 and heated over a hot plate. Similar fractions were pooled together to yield 21 major fractions (fractions B-I to B-XXI).

Fraction B-VIII, which was eluted with *n*-hexane/ CH_2Cl_2 (2:8), yielded 10 subfractions. Subfractions 1–4 were pooled together (0.2 g) and were further purified by PTLC using the solvent system *n*-hexane/EtOAc (7:3) to yield compound 4 (3 mg). Fraction B-IX, eluted with CH_2Cl_2 (100%), yielded 57 subfractions. Subfractions 2–15 were

pooled together (0.25 g) and were further purified by PTLC using *n*-hexane/EtOAc (6:4) to yield compounds 1 (3 mg) and 2 (8 mg). Subfractions 47 and 48 obtained from fraction B-IX were pooled together (0.11 g) and were further purified on PTLC using *n*-hexane/EtOAc (6:4) to yield 3 mg of compound 3. Fraction B-XI was eluted with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (98:2) and yielded 31 subfractions. Subfraction 22 afforded 10 mg of compound 5 by crystallization from $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (98:2). Fraction B-XII was eluted with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (97:3) and yielded 34 subfractions. Subfraction 12 (0.06 g) afforded 10 mg of compound 6 by crystallization from $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (97:3). Similarly, subfraction 15 (0.17 g) afforded 10 mg of compound 7 by crystallization from $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (97:3). The supernatant was evaporated until dryness and was further crystallized with MeOH to yield 30 mg of compound 8.

(2*R*,3*R*)-3,5,7-Trihydroxy-8-methoxyflavanone (1): $[\alpha]_D^{25} -23$ (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log *e*) 294 (4.32), 340sh (3.65); ECD (*c* 18 mM, MeOH) λ_{max} ($\Delta\epsilon_{\text{max}}$) 303 (−0.048), 370 (+0.004) nm; IR (KBr) ν_{max} 3385 (OH), 1722 (C=O), 1582 (benzene ring) cm^{-1} ; ^1H NMR (400 MHz, pyridine- d_5) and ^{13}C NMR (100 MHz, pyridine- d_5), see Table 1; HRESIMS *m/z* 303.0860 [*M* + *H*⁺] (calcd for $\text{C}_{16}\text{H}_{15}\text{O}_6$, 303.0863).

Cell Culture and Cell Viability Assay. The mucosal mast-cell-derived rat basophilic leukemia cells (RBL-2H3) were obtained from Bioresource Collection and Research Center (Hsin-Chu, Taiwan). The cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin in 10 cm cell culture dishes at 37°C in a humidified chamber with 5% CO_2 in air. The potential cytotoxic effects of the samples (10 to 100 $\mu\text{g}/\text{mL}$ for the crude extract and 1 to 400 μM for the pure compounds) on RBL-2H3 cells were determined by the methylthiazole tetrazolium (MTT) assay as described previously.⁶¹

Degranulation Assay in Mast Cells. The level of degranulation in mast cells was evaluated based on A23187- and antigen-induced β -hexosaminidase release in RBL-2H3 cells according to a reported method.⁶¹ Briefly, the cells were seeded in a 96-well plate (2×10^4 cells/well, for the A23187-induced assay) or a 48-well plate (3×10^4 cells/well, for the antigen-induced assay) overnight. RBL-2H3 cells were treated with the samples (10 to 100 $\mu\text{g}/\text{mL}$ for the crude and 1 to 400 μM for the pure compounds) for 20 h. For the A23187-induced assay, the cells were activated by A23187 (1 μM), the calcium ionophore. For the antigen-induced assay, the cells were first sensitized with anti-DNP IgE (1 $\mu\text{g}/\text{mL}$, at least 2 h) and then activated by DNP-BSA (100 ng/mL) for 1 h. Dexamethasone (50 nM) served as the positive control. The amount of β -hexosaminidase was detected using the method utilizing *p*-NAG as the substrate according to the procedure described before.⁶¹

Superoxide Anion Generation and Elastase Release Assays by Human Neutrophils. The human neutrophils were obtained from venous blood of healthy adult volunteers (20–30 years old) following the reported procedure.⁶² Measurement of superoxide anion generation by the activated neutrophils was based on the reduction of ferricytochrome *c* as described before.^{62,63} Elastase release by the activated neutrophils was determined using *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide as the elastase substrate according to the reported method.^{62,63} The concentration was 1 to 10 $\mu\text{g}/\text{mL}$ for the crude samples and 0.3 to 10 μM for the pure compounds. LY294002 was used as the positive control.

Molecular Docking. The potential molecular binding mode between the isolated compounds and HNE was evaluated using the CDocker algorithm in Discovery Studio 2.5 (Accelrys Software, Inc., San Diego, CA, USA). The crystal structure of HNE (PDB ID: 1H1B) was retrieved from the Protein Data Bank (<http://www.rcsb.org/pdb/>).⁶⁴ Water molecules in the protein were removed except for those involved in the binding of the inhibitor, and the protein was refined. The cocrystallized ligand GW475151 was used as the positive control ligand, and the binding site was defined based on the binding of GW475151 and HNE. Prior to docking, the cocrystallized ligand was removed; then, the prepared ligands were docked to the protein using appropriate parameters. The interaction energy was calculated to analyze the interaction between the ligand and the receptor. For each

ligand, the top 10 ligand-binding poses were ranked according to their CDocker energies, and the predicted binding interactions were analyzed, from which the best ligand-binding poses were chosen. Validation of CDocker as a docking algorithm for HNE was achieved by calculating the root-mean-square deviations (RMSD) after redocking the cocrystallized ligand to the protein structure using the algorithm.

Statistics. The results were expressed as mean $\pm$ SD (antiallergic assay) or mean $\pm$ SEM (anti-inflammatory assays). The IC₅₀ values were calculated using Microsoft Office (linear function, antiallergic assay) or SigmaPlot (anti-inflammatory assays). Statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test (SigmaPlot, Systat Software Inc., San Jose, CA, USA). Values with * $P < 0.05$, ** $P < 0.001$ were considered statistically significant.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.jnatprod.7b00476](https://doi.org/10.1021/acs.jnatprod.7b00476).

¹H NMR, ¹³C NMR, 2D NMR, HRESIMS data of compound 1 and ¹H NMR, ¹³C NMR, 2D NMR, ESIMS spectra of compound 2 (PDF)

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Notes

The authors declare no competing financial interest.

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**Probing the Anti-allergic and Anti-inflammatory Activity of Biflavonoids and
Dihydroflavonols from *Dietes bicolor* (Iridaceae)**

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Spectroscopic data of compounds 2-8:

3-Hydroxy-5-methoxy-6,7-methylenedioxyflavanone (**2**): $^1\text{H-NMR}$ (400 MHz, CD_3OD): 7.51 (2H, dd, $J = 7.2, 2.0$ Hz, H-2', 6'), 7.43-7.37 (3H, m, H-3', 4', 5'), 6.26 (1H, s, H-8), 5.99 (2H, s, O-CH₂-O), 5.02 (1H, d, $J = 12$ Hz, H-2), 4.42 (1H, d, $J = 12$ Hz, H-3), 4.06 (3H, s, 5-OCH₃). $^1\text{H-NMR}$ (500 MHz, pyridine-*d*₅): 7.78 (2H, dd, $J = 7.0, 2.0$ Hz, H-2', 6'), 7.47-7.37 (3H, m, H-3', 4', 5'), 6.44 (1H, s, H-8), 5.96 (2H, d, $J = 5$ Hz, O-CH₂-O), 5.40 (1H, d, $J = 11.5$ Hz, H-2), 4.83 (1H, d, $J = 11.5$ Hz, H-3), 4.10 (3H, s, 5-OCH₃). $^{13}\text{C-NMR}$ (125 MHz, pyridine-*d*₅): 191.7 (C-4), 160.2 (C-9), 155.2 (C-7), 143.1 (C-5), 138.4 (C-1'), 132.7 (C-6), 129.1 (C-4'), 128.8 (C-3', 5'), 128.5 (C-2', 6'), 107.4 (C-10), 102.4 (O-CH₂-O), 93.6 (C-8), 84.4 (C-2), 74.3 (C-3), 60.4 (5-OCH₃); ESIMS m/z 315.13 $[\text{M}+\text{H}]^+$ consistent with the molecular formula $\text{C}_{17}\text{H}_{14}\text{O}_6$.

3-Hydroxy-5,7-dimethoxy flavanone (**3**): $^1\text{H-NMR}$ (400 MHz, CD_3OD) 7.51-7.49 (2H, m, H-2', 6'), 7.38-7.30 (3H, m, H-3', 4', 5'), 6.21 (1H, d, $J = 2.4$ Hz, H-8), 6.15 (1H, d, $J = 2.4$ Hz, H-6), 5.03 (1H, d, $J = 12.0$ Hz, H-2), 4.39 (1H, d, $J = 12.0$ Hz, H-3), 3.84 (3H, s, 5-OCH₃), 3.81 (3H, s, 7-OCH₃).

Orobol 7,3'-dimethyl ether (**4**): $^1\text{H-NMR}$ (600 MHz, CD_3OD): 8.17 (1H, s, H-2), 7.18 (1H, d, $J = 1.8$ Hz, H-2'), 6.98 (1H, dd, $J = 7.8, 1.8$ Hz, H-6'), 6.85 (1H, d, $J = 7.8$ Hz, H-5'), 6.56 (1H, d, $J = 2.4$ Hz, H-8), 6.38 (1H, d, $J = 2.4$ Hz, H-6), 3.90 (3H, s, 3'-OCH₃), 3.89 (3H, s, 7-OCH₃). $^{13}\text{C-NMR}$ (200 MHz, CD_3OD) (δ ppm): 182.0 (C-4), 167.2 (C-7), 161.0 (C-5), 159.8 (C-9), 154.2 (C-2), 148.8 (C-3'), 146.9 (C-4'), 125.1 (C-3), 123.7 (C-1'), 122.9 (C-6'), 116.2 (C-5'), 114.0 (C-2'), 105.0 (C-10), 99.28 (C-6), 93.28 (C-8), 57.3 (3'-OCH₃), 56.5 (7-OCH₃); ESIMS m/z 314.98 $[\text{M}+\text{H}]^+$ consistent with the molecular formula $\text{C}_{17}\text{H}_{14}\text{O}_6$.

Lanaroflavone (**5**): UV (MeOH) λ_{max} (nm) 270, 342 nm; $^1\text{H NMR}$ (400 MHz, CD_3OD): 7.88 (2H, d, $J = 8.0$ Hz, H-2', 6'), 7.81 (2H, d, $J = 8.0$ Hz, H-2'', 6''), 7.04 (2H, d, $J = 8.0$ Hz, H-3', 5'), 6.89 (2H, d, $J = 8.0$ Hz, H-3'', 5''), 6.61 (1H, s, H-3), 6.51 (1H, s, H-3''), 6.44 (1H, s, H-6''), 6.42 (1H, d, $J = 2.0$ Hz, H-8), 6.16 (1H, d, $J = 2.0$ Hz, H-6).

Robustaflavone (**6**): $^1\text{H-NMR}$ (400 MHz, CD_3OD): 8.12 (1H, d, $J = 2.4$ Hz, H-2'), 7.91 (1H, dd, $J = 8.8, 2.4$ Hz, H-6'), 7.61 (2H, d, $J = 8.8$ Hz, H-2'', 6''), 7.11 (1H, d, $J = 8.8$ Hz, H-5'), 6.66 (2H, d, $J = 8.8$ Hz, H-3'', 5''), 6.64 (1H, s, H-3'''), 6.62 (1H, s, H-3), 6.32 (1H, s, H-8''), 6.27 (1H, d, $J = 2.0$ Hz, H-8), 6.17 (1H, d, $J = 2.0$ Hz, H-6); $^{13}\text{C-NMR}$ (100 MHz, CD_3OD): 184.1 (C-4), 183.8 (C-4''), 166.5 (C-7), 166.2 (C-7''), 165.7 (C-2, 2''), 163.1 (C-5, 5''), 162.5 (C-4'), 162.4 (C-4'''), 159.4 (C-9, 9''), 132.8 (C-2'), 129.3 (C-2'', 6''), 128.3 (C-6'), 123.3 (C-1''), 122.5 (C-3'), 122.2 (C-1'), 119.2 (C-5'), 116.8 (C-3'', 5''), 107.1 (C-6''), 105.2 (C-10), 104.6 (C-10''), 103.7 (C-3), 103.2 (C-3''), 100.2 (C-6), 95.1 (C-8, 8''); ESIMS m/z 539.22 $[\text{M}+\text{H}]^+$ corresponding to the molecular formula $\text{C}_{30}\text{H}_{18}\text{O}_{10}$.

Amentoflavone (**7**): UV (MeOH) λ_{max} (nm) 270, 336; NaOMe 277, 397; AlCl_3 279, 300sh, 339, 346, 384; $\text{AlCl}_3 + \text{HCl}$ 276, 297sh, 340; NaOAc 275, 371; NaOAc + H_3BO_3 272, 328; $^1\text{H-NMR}$ (400 MHz, pyridine-*d*₅): 8.58 (1H, d, $J = 2.4$ Hz, H-2'), 7.91 (1H, dd, $J = 8.4, 1.6$ Hz, H-6'), 7.90 (2H, d, $J = 8.8$ Hz, H-2'', 6''), 7.41 (1H, d, $J = 8.4$ Hz, H-5'), 7.13 (2H, d, $J = 8.8$ Hz, H-3'', 5''), 7.05 (1H, s, H-3'''), 6.93 (1H, s, H-3), 6.90 (1H, s, H-6''), 6.79 (1H, d, $J = 2.4$ Hz, H-8), 6.73 (1H, d, $J = 2.4$ Hz, H-6); $^{13}\text{C-NMR}$ (100 MHz, pyridine-*d*₅): 183.0 (C-4''), 182.7 (C-4), 165.8 (C-2), 164.9 (C-2''), 164.4 (C-7), 163.1 (C-7''), 162.6 (C-5), 162.4 (C-5''), 162.2 (C-4'''), 162.0 (C-4'), 158.5 (C-9), 155.9 (C-9''), 132.5 (C-2'), 128.8 (C-2'', 6''), 128.1 (C-6'), 122.5 (C-3'), 122.3 (C-1'),

121.8 (C-1'''), 117.8 (C-5'), 116.8 (C-3''', 5'''), 105.8 (C-8''), 104.9 (C-10''), 104.7 (C-10), 103.9 (C-3), 103.5 (C-3''), 100.4 (C-6''), 100.0 (C-6), 94.8 (C-8); ESIMS m/z 539.22 $[M+H]^+$ consistent with the molecular formula $C_{30}H_{48}O_{10}$.

β -Sitosterol (**8**): ^1H -NMR (400 MHz, $CDCl_3$): 0.68 (3H, s, H-18), 0.80 (3H, t, $J = 6.8$ Hz, H-29), 0.82 (3H, d, $J = 7.2$ Hz, H-27), 0.84 (3H, d, $J = 7.2$ Hz, H-26), 0.91 (3H, d, $J = 6.8$ Hz, H-21), 1.01 (3H, s, H-19), 3.56 (1H, m, H-3), 5.30 (1H, m, H-6).

Figure S1. ^1H NMR spectrum of 3,5,7-trihydroxy-8-methoxyflavanone (CDCl_3) (**1**)

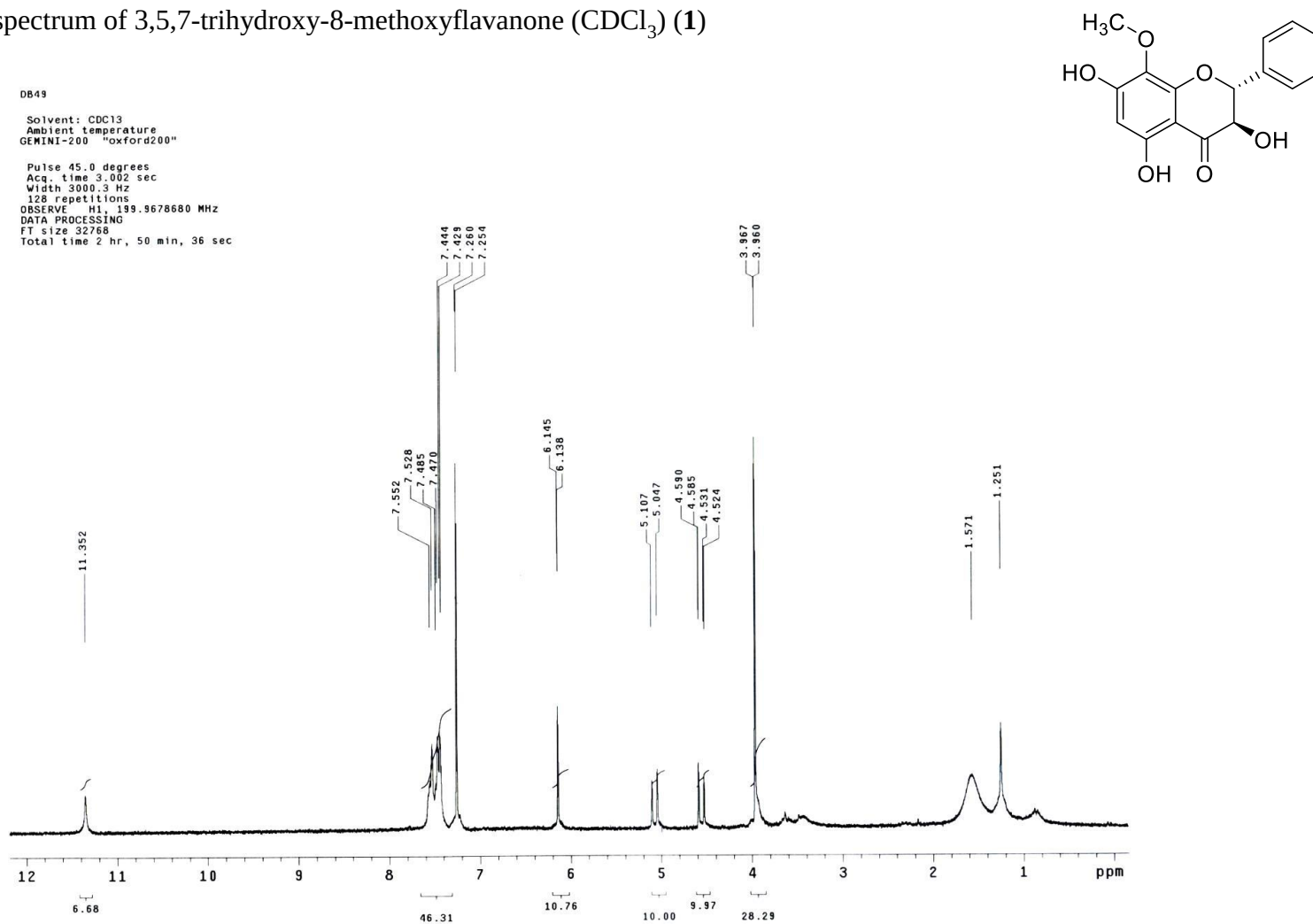


Figure S2. ^1H NMR spectrum of 3,5,7-trihydroxy-8-methoxyflavanone (**1**) (pyridine- d_5)

DB-49
Pulse Sequence: s2pul
UNITYplus-400 "unity400"
Date: Sep 4 2012
Solvent: pyridine
Ambient temperature
Total 108 repetitions

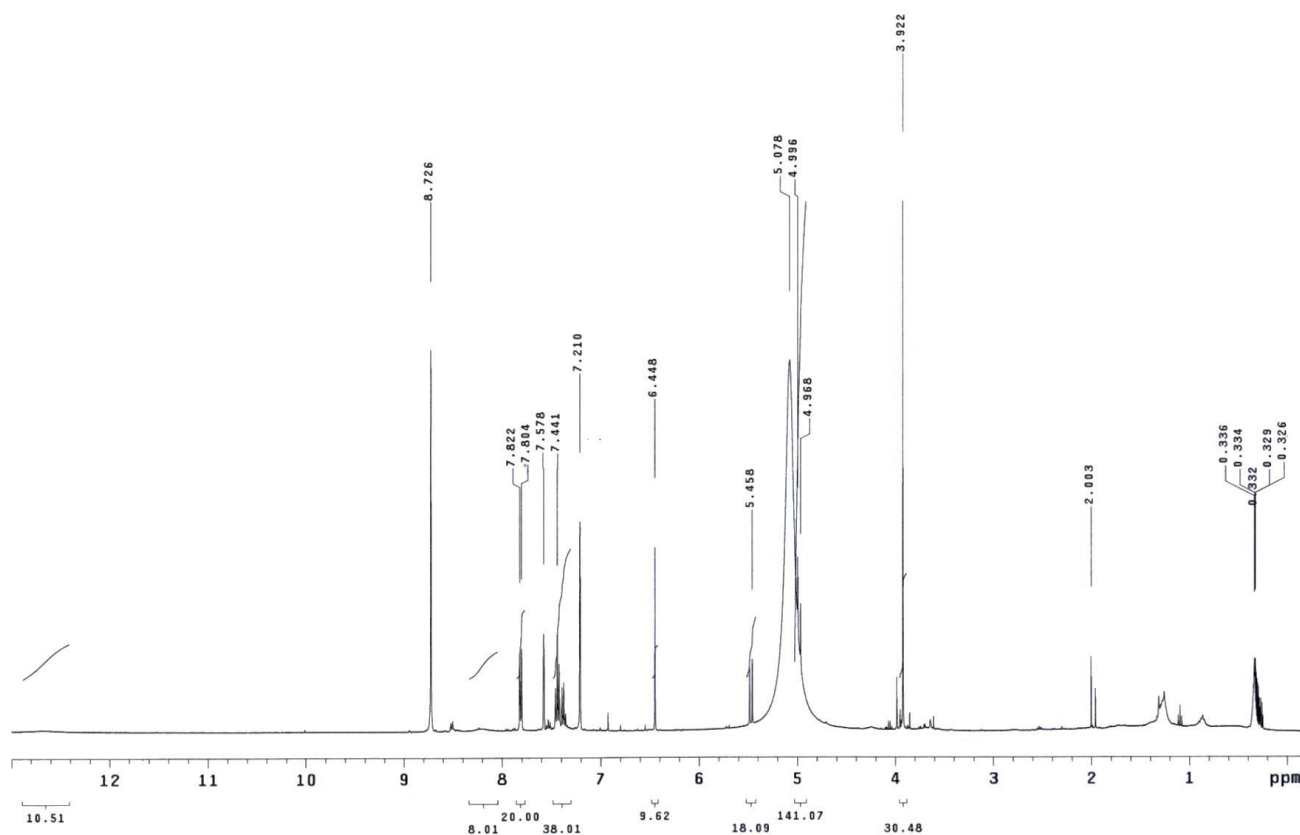
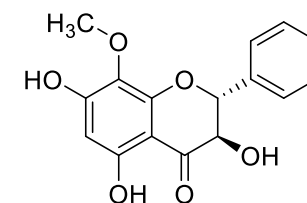


Figure S3. ^{13}C NMR spectrum of 3,5,7-trihydroxy-8-methoxyflavanone (**1**) (pyridine- d_5)

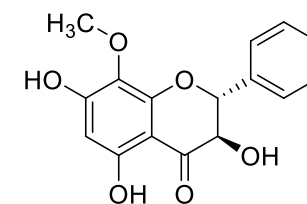
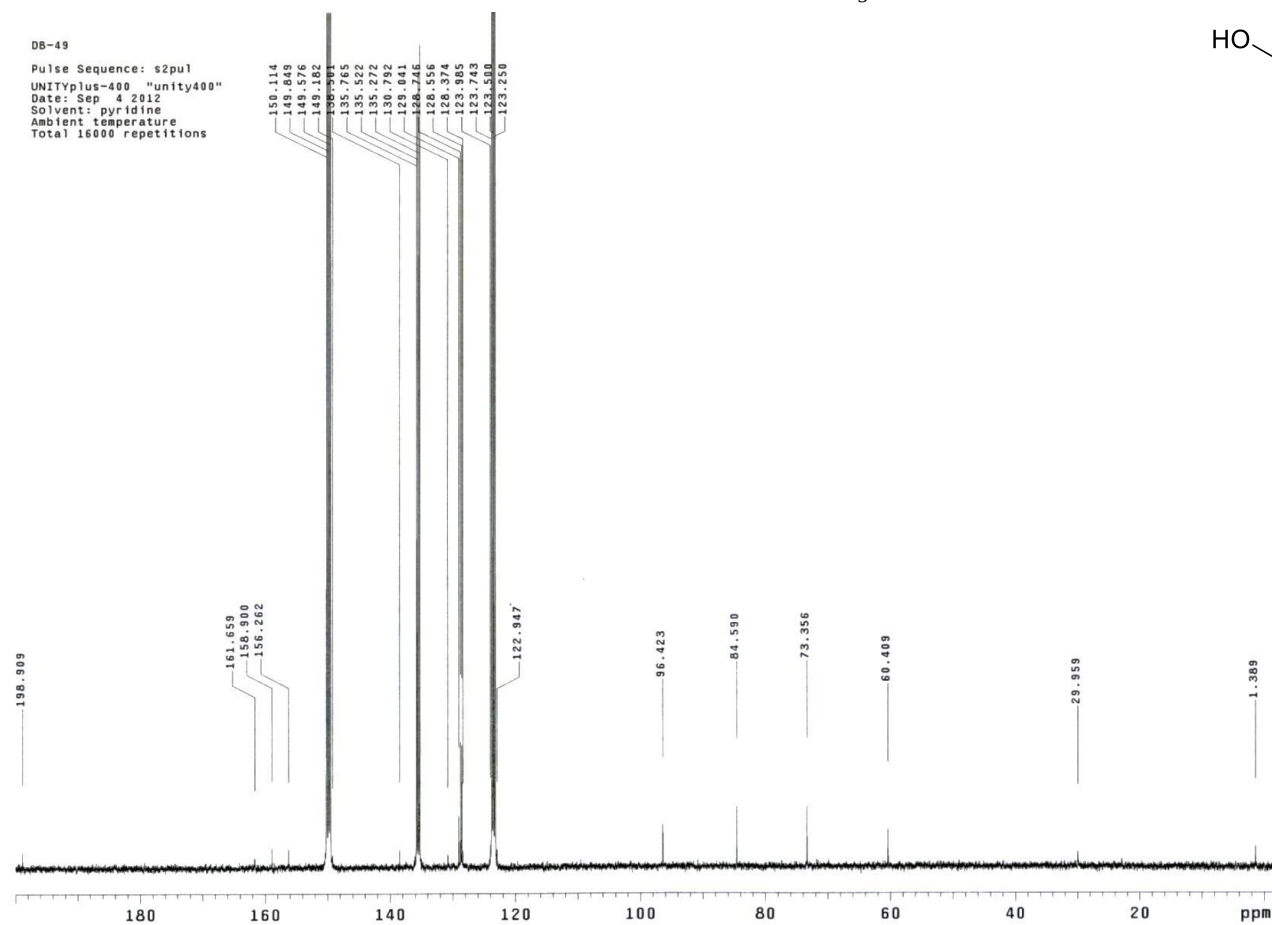


Figure S4. DEPT spectrum of 3,5,7-trihydroxy-8-methoxyflavanone (**1**) (pyridine- d_5)

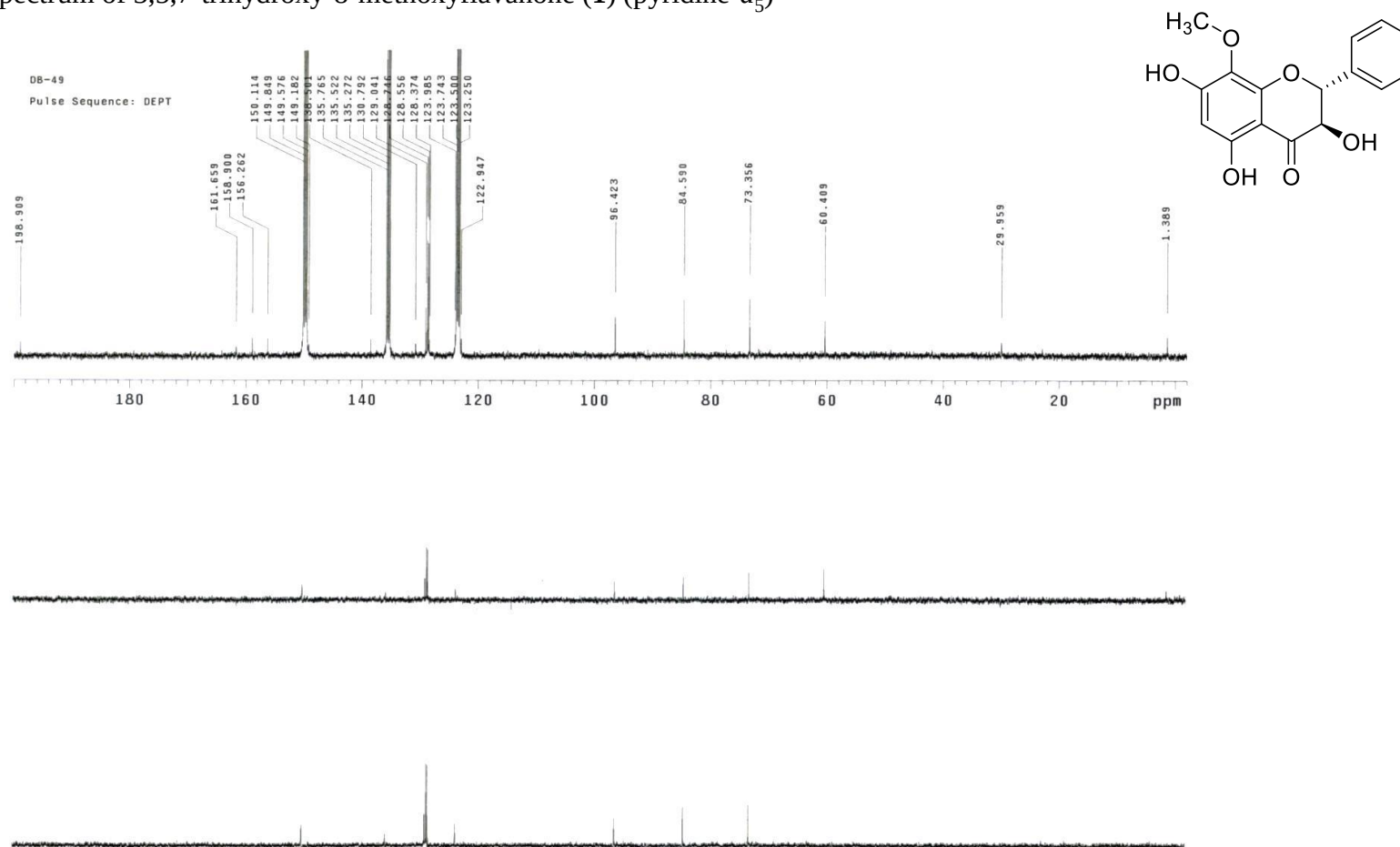


Figure S5. HSQC spectrum of 3,5,7-trihydroxy-8-methoxyflavanone (**1**) (pyridine- d_5)

DB-49
Pulse Sequence: gHSQC

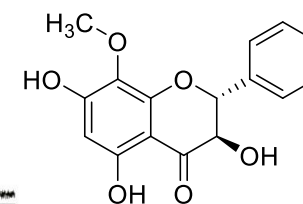
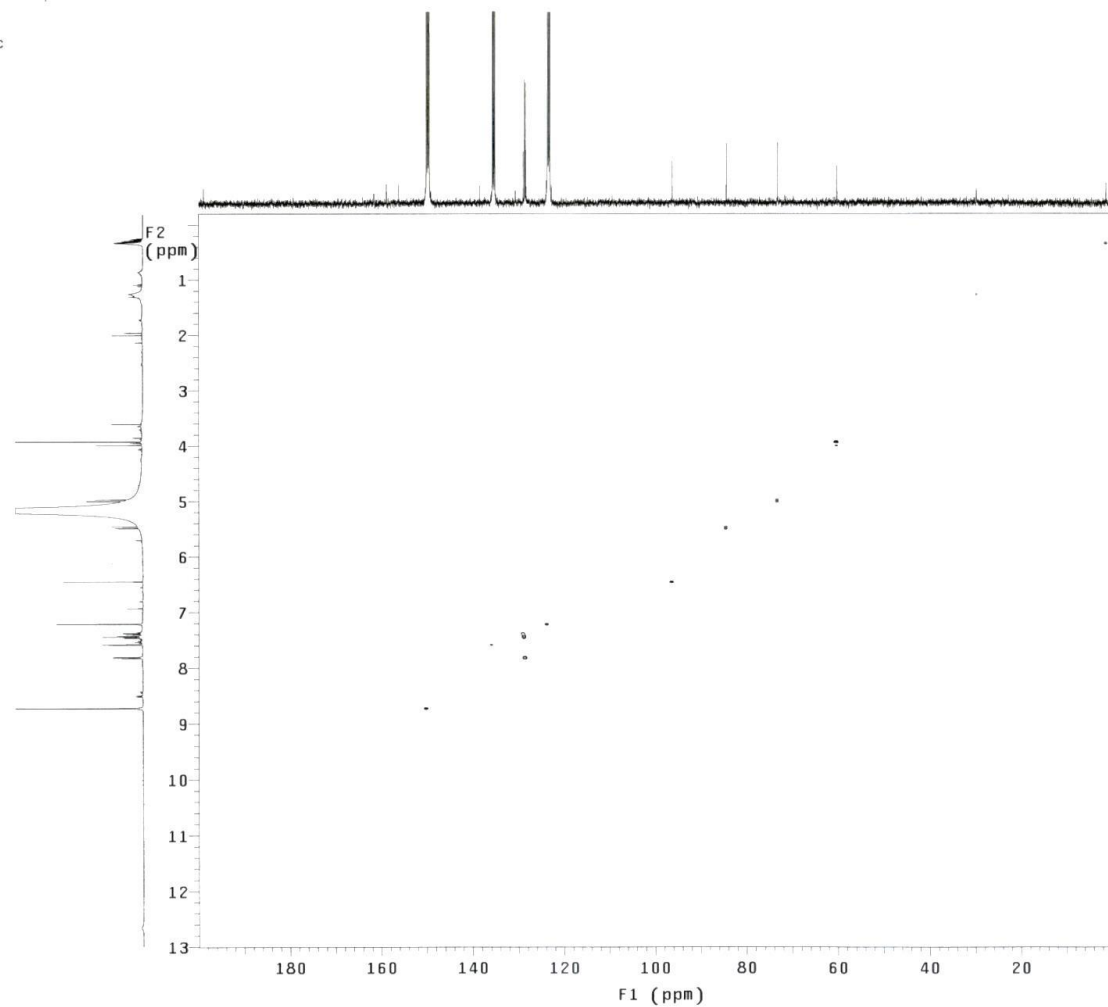
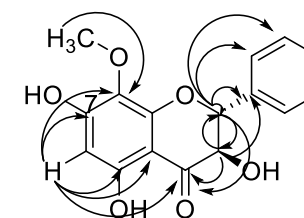
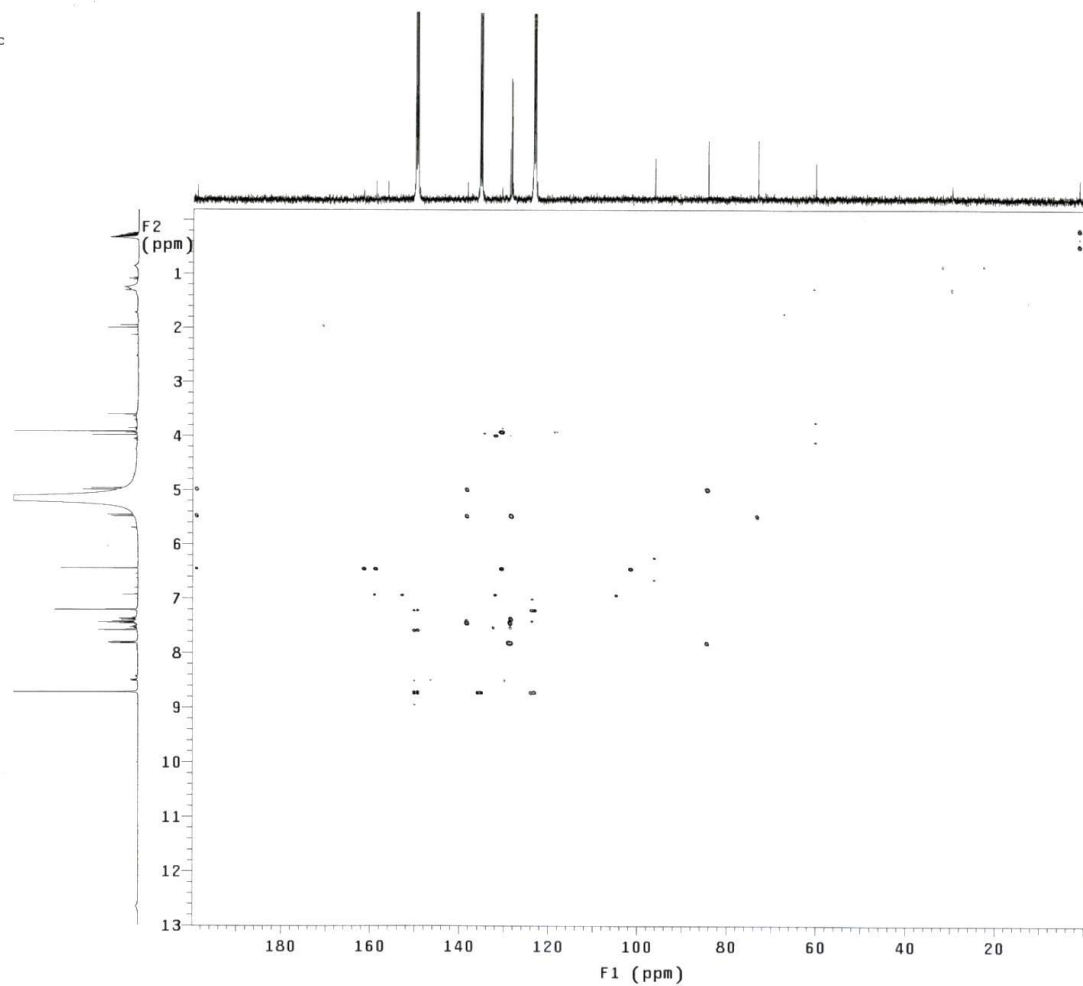


Figure S6. HMBC spectrum of 3,5,7-trihydroxy-8-methoxyflavanone (**1**) (pyridine- d_5)

DB-49
Pulse Sequence: gHMBC



DB-49
Pulse Sequence: gHMBC

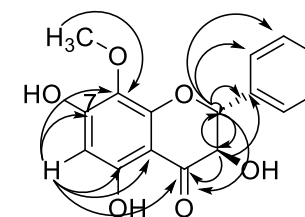
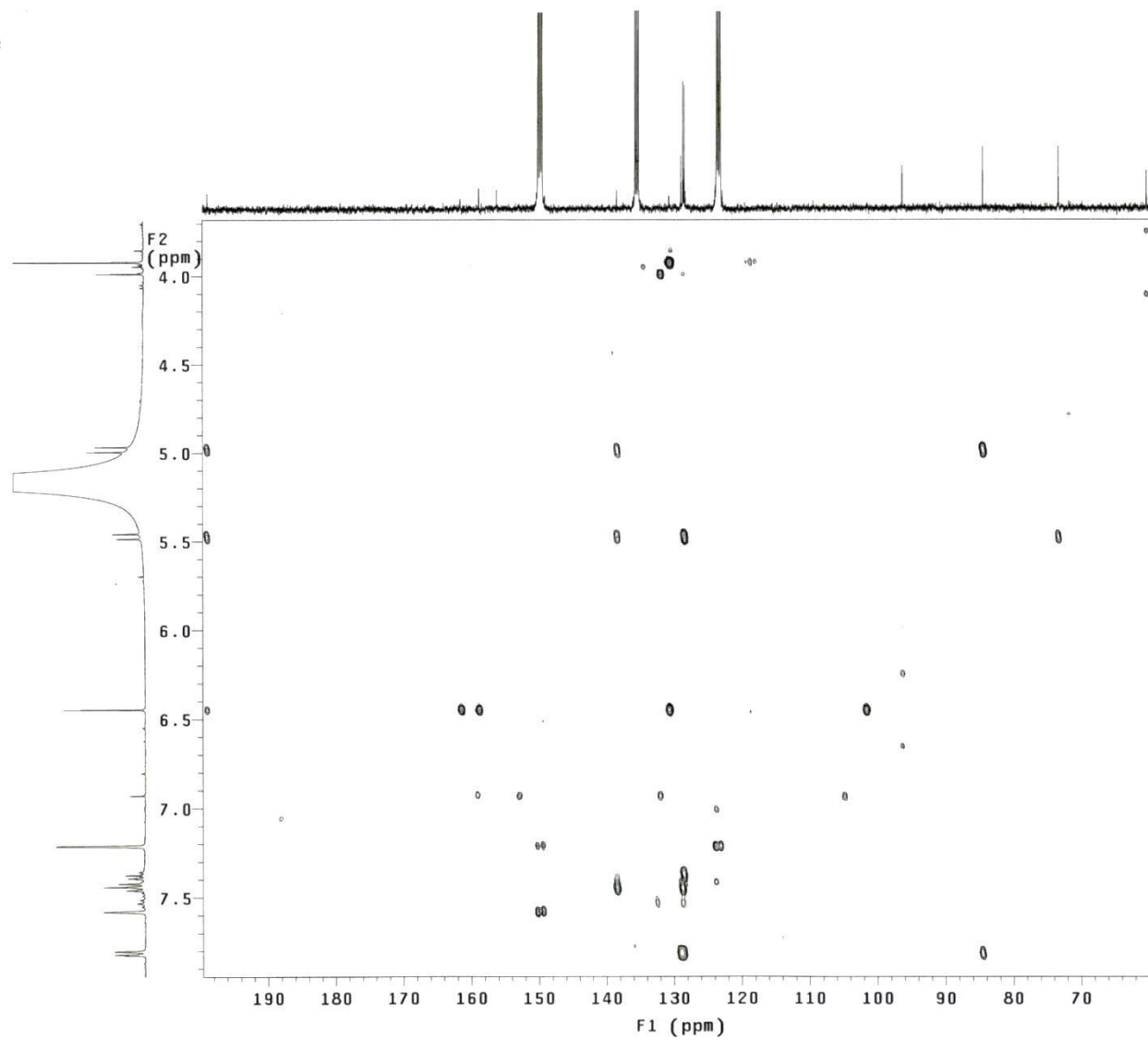


Figure S7. ^1H , ^1H COSY spectrum of 3,5,7-trihydroxy-8-methoxyflavanone (**1**) (pyridine- d_5)

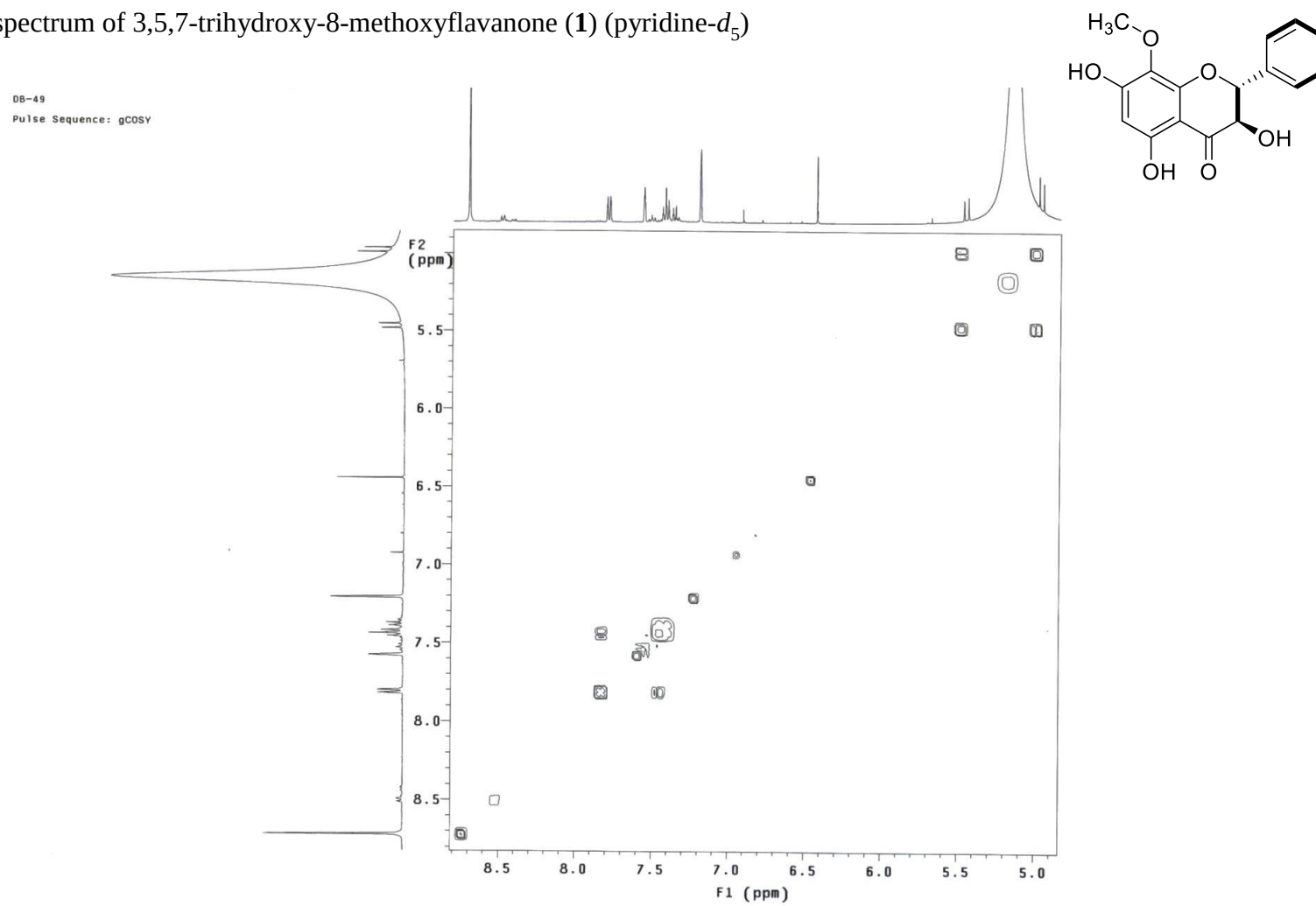


Figure S8. NOESY spectrum of 3,5,7-trihydroxy-8-methoxyflavanone (**1**) (pyridine- d_5)

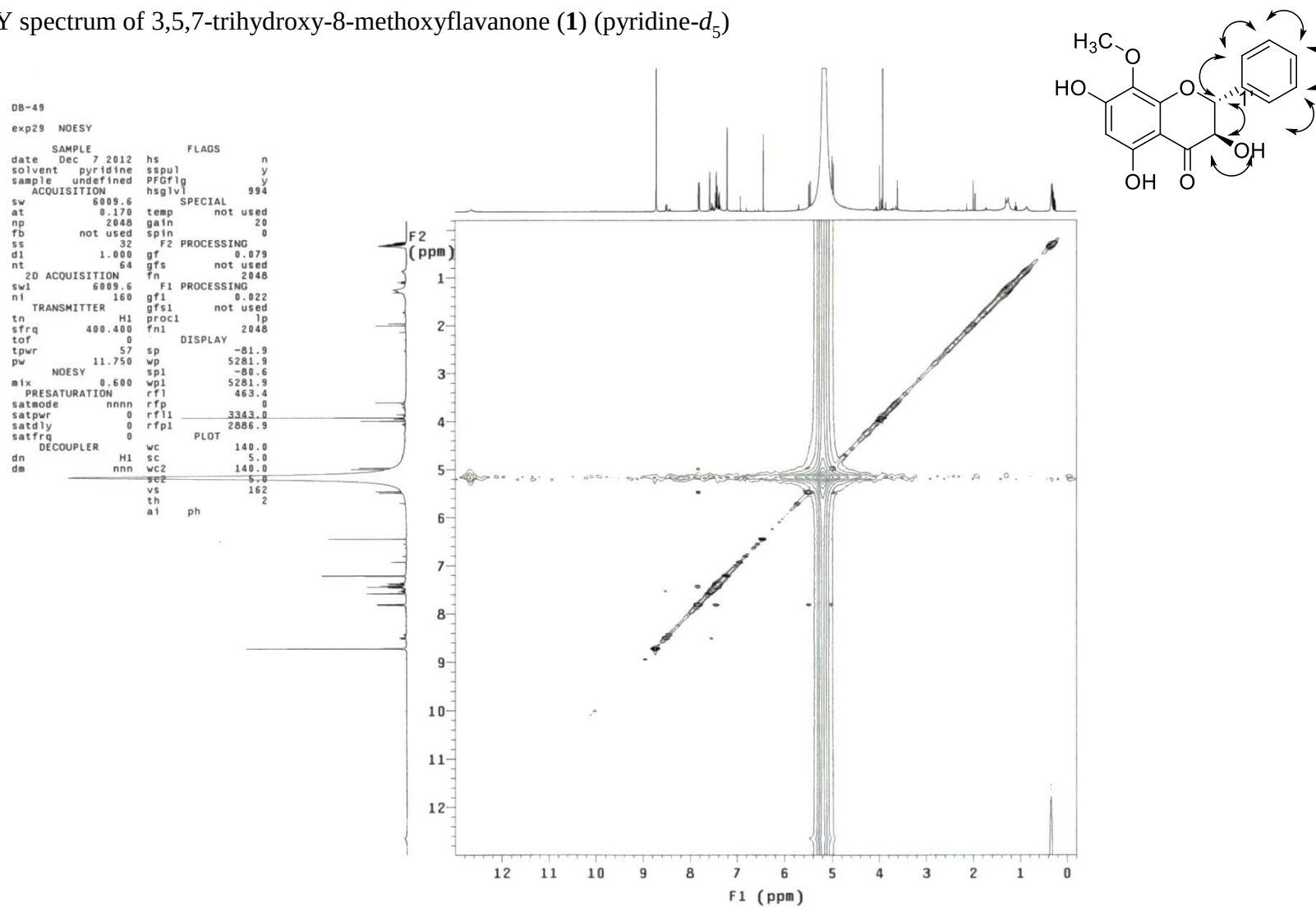


Figure S9. HRESIMS spectrum of 3,5,7-trihydroxy-8-methoxyflavanone (**1**)

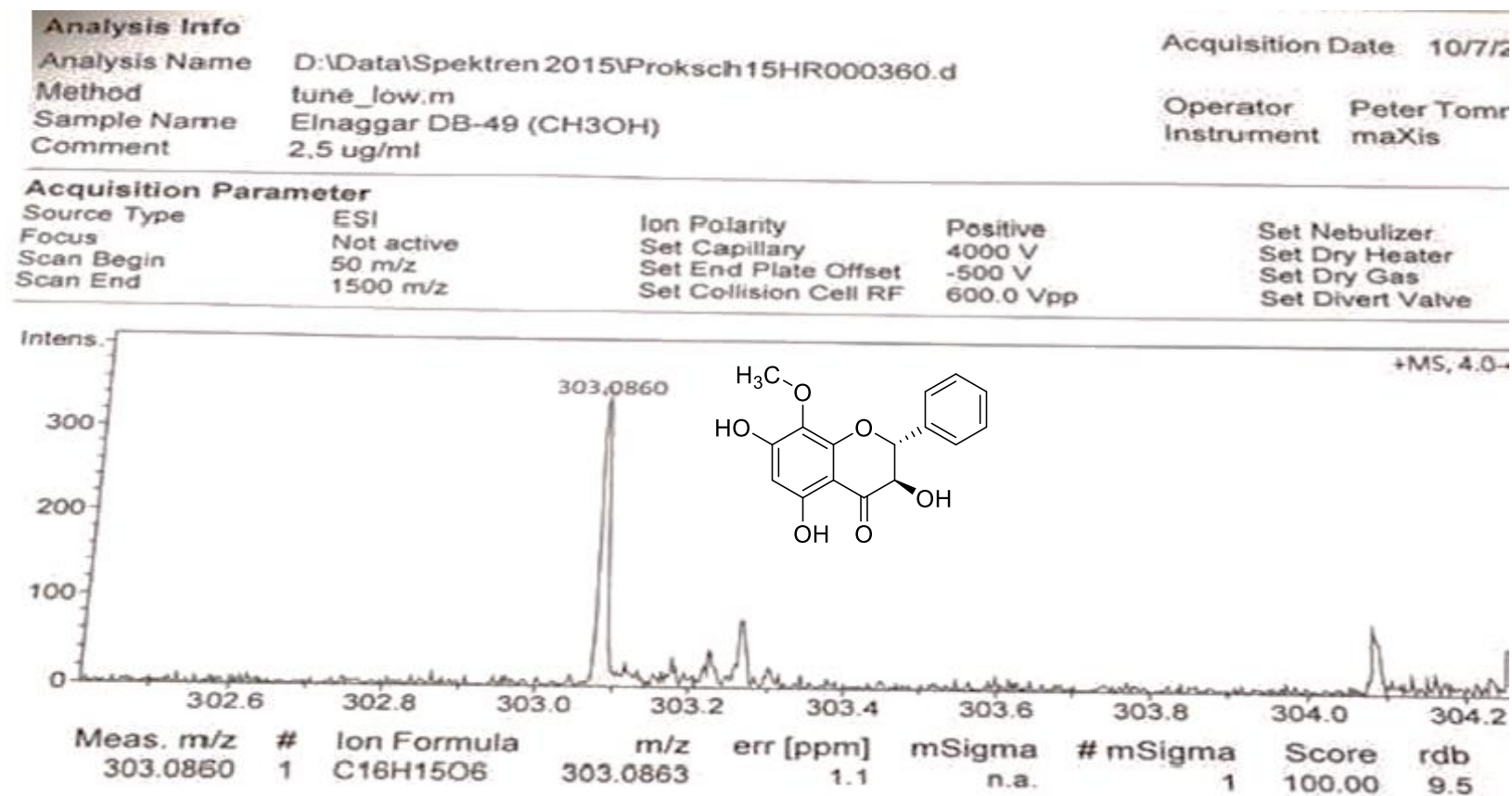


Figure S10. ESIMS spectrum of 3,5,7-trihydroxy-8-methoxyflavanone (**1**)

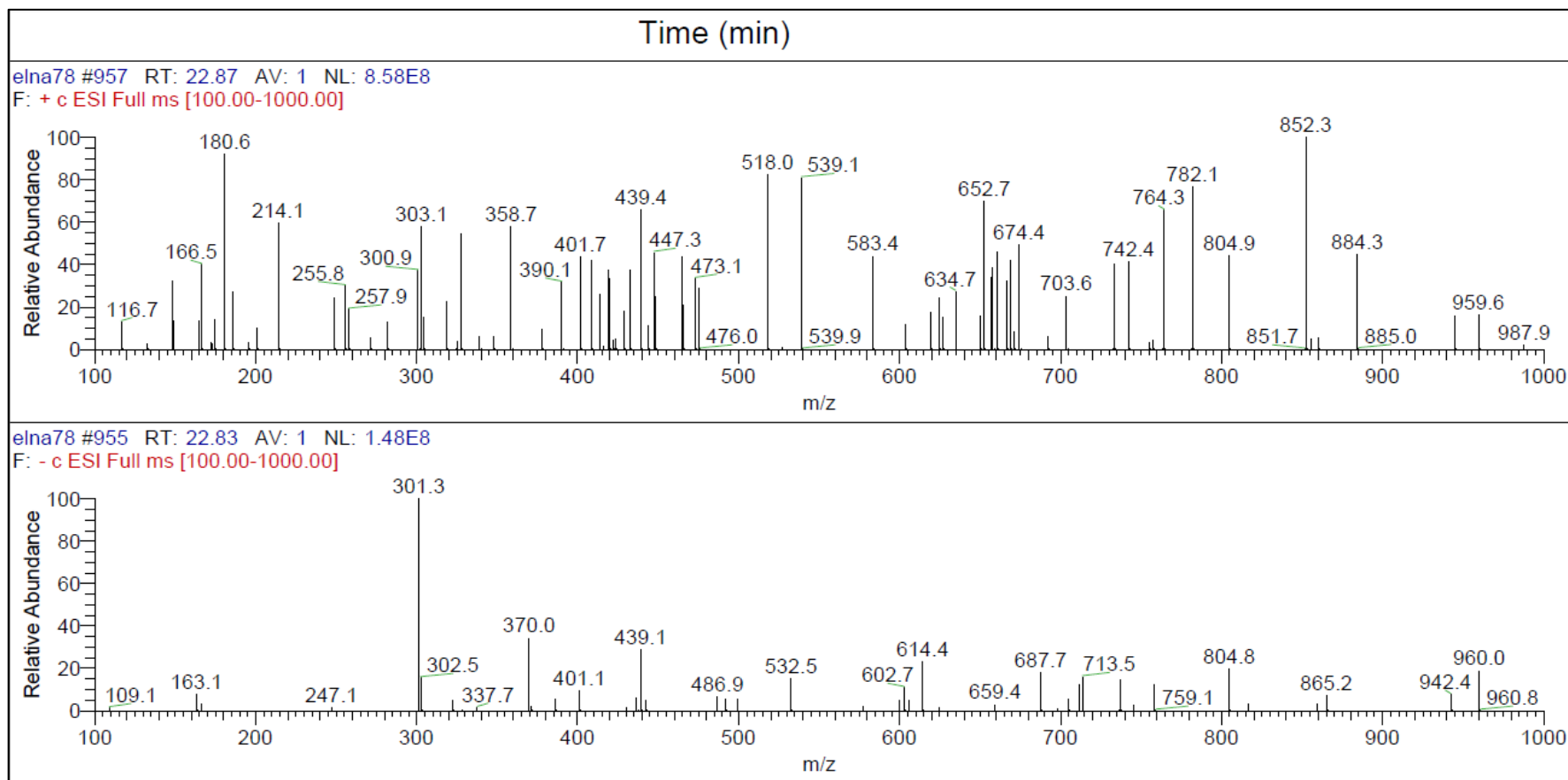
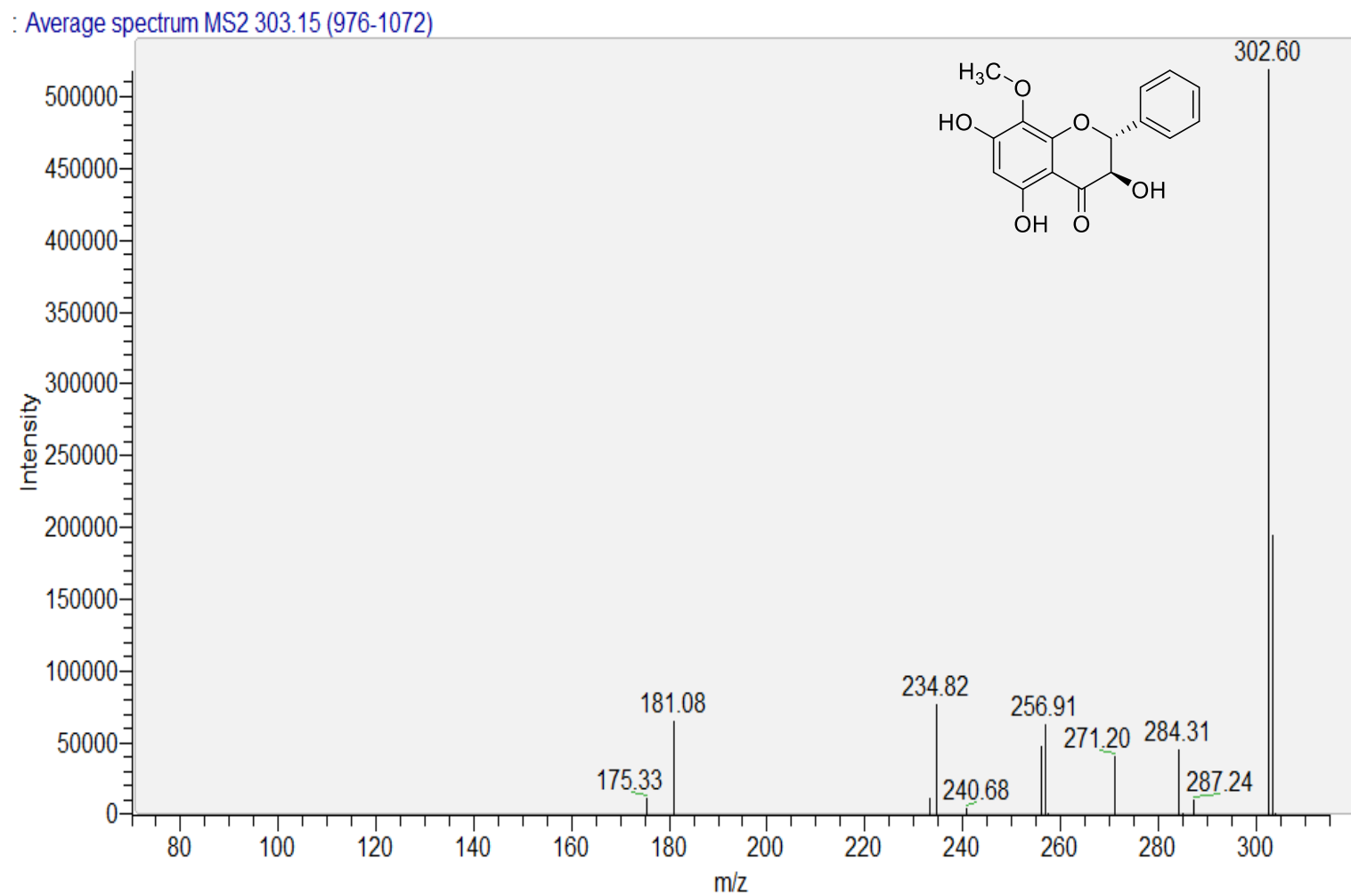
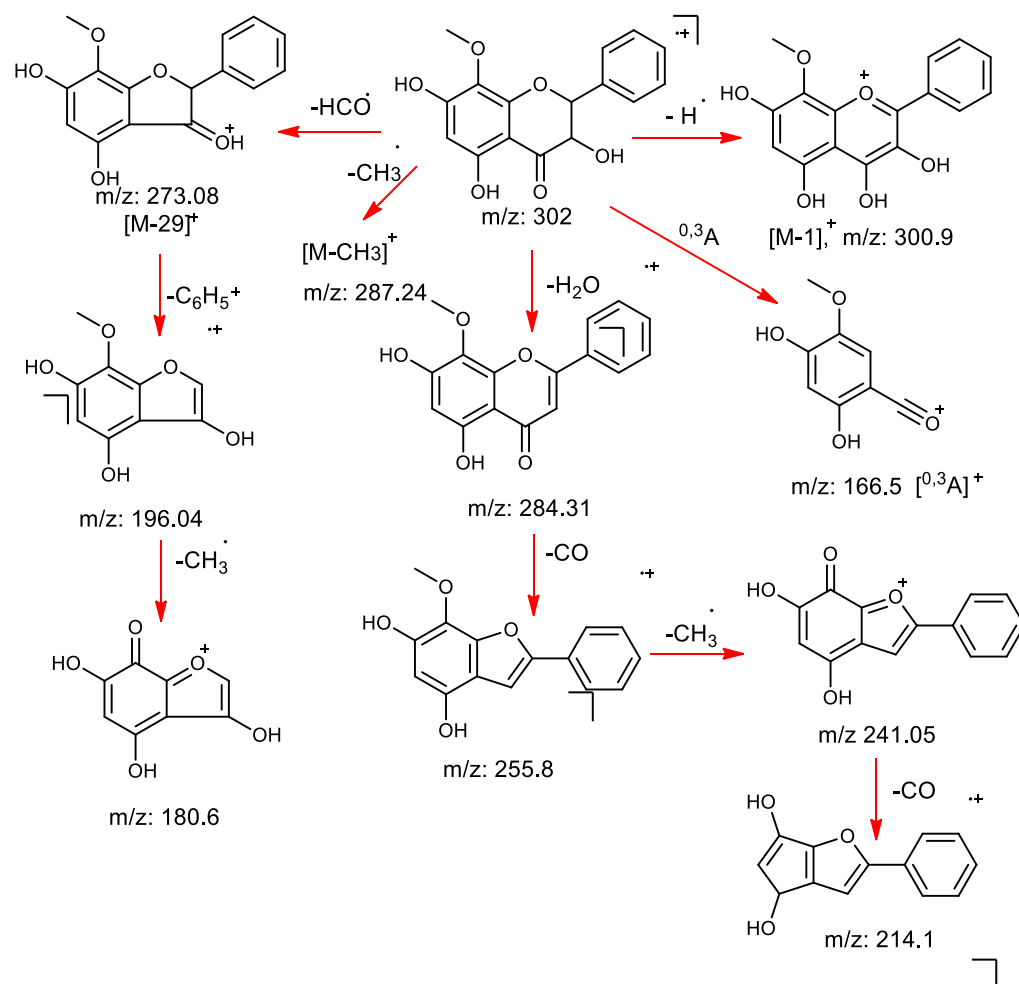


Figure S11. ESIMS² fragments of 3,5,7-trihydroxy-8-methoxyflavanone (**1**)





ESIMS spectrum (Figure S10) displayed fragment ion peaks at m/z 300.9 $[\text{M}-1]^+$, m/z 255.8 $[\text{M}-\text{H}_2\text{O}-\text{CO}]^+$, m/z 214.1 $[\text{C}_{13}\text{H}_{10}\text{O}_3]^+$, m/z 180.6 $[\text{C}_8\text{H}_5\text{O}_5]^+$ and m/z 166.5 $^{[0,3A]}+$ which was in agreement with the fragmentation pattern of dihydroflavonols.

Figure S12. IR spectrum of 3,5,7-trihydroxy-8-methoxyflavanone (**1**)

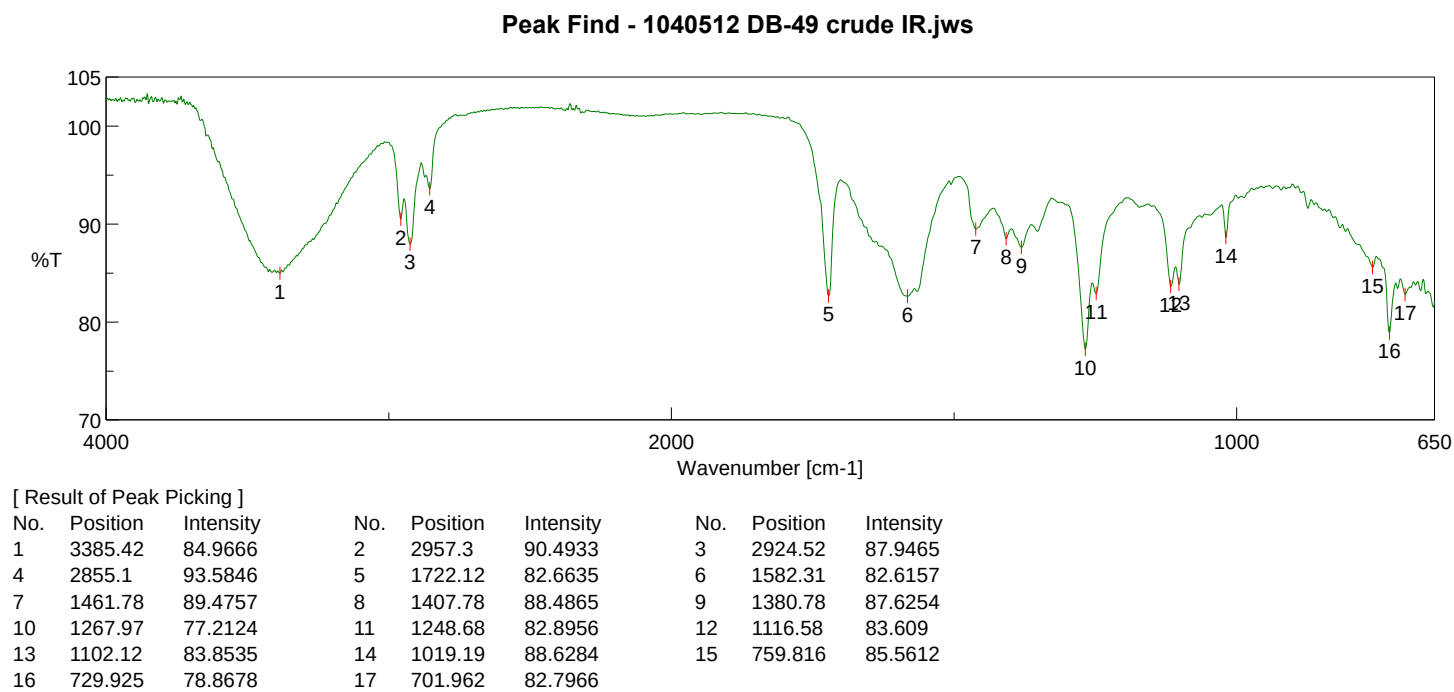


Figure S13. CD spectrum of 3,5,7-trihydroxy-8-methoxyflavanone (**1**)

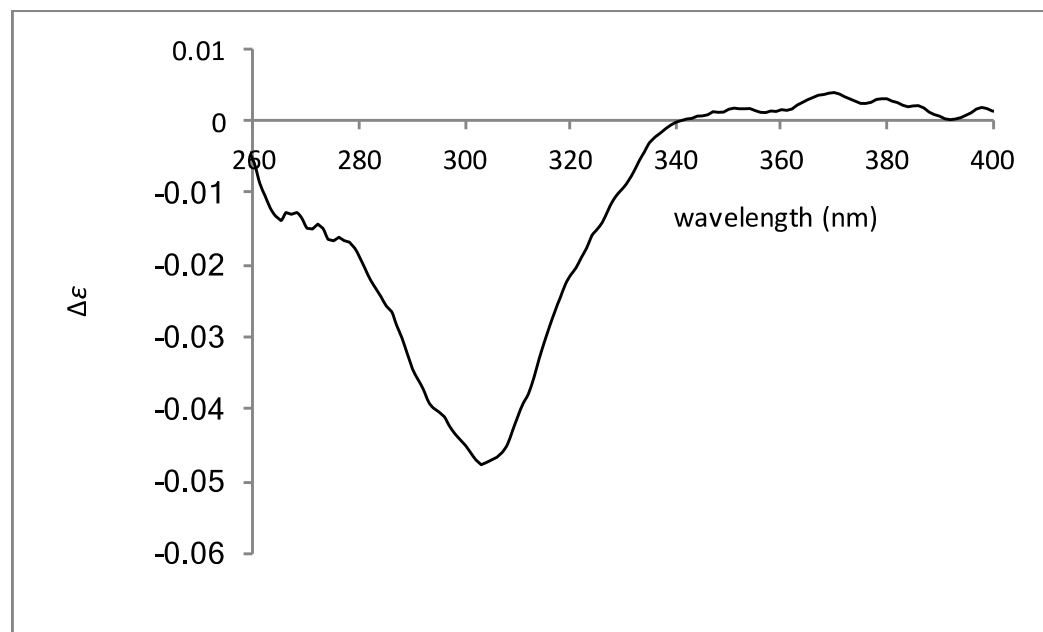


Figure S14. UV-Vis absorption spectrum for of 3,5,7-trihydroxy-8-methoxyflavanone (**1**) in methanol (A) and in presence of AlCl_3 (B)

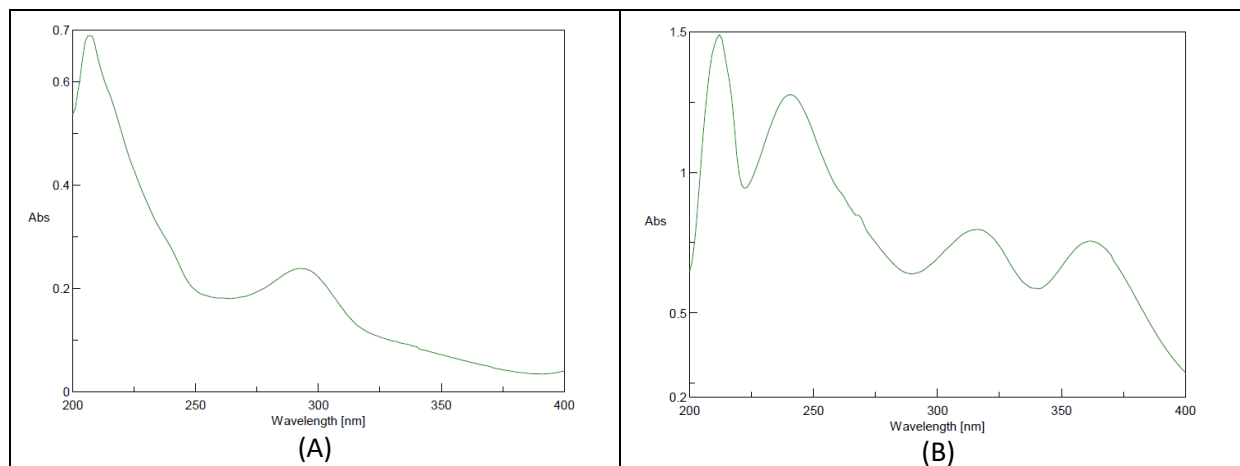


Figure S15. ^1H NMR spectrum of 3-hydroxy-5-methoxy-6,7-methylenedioxyflavanone (**2**) in CD_3OD

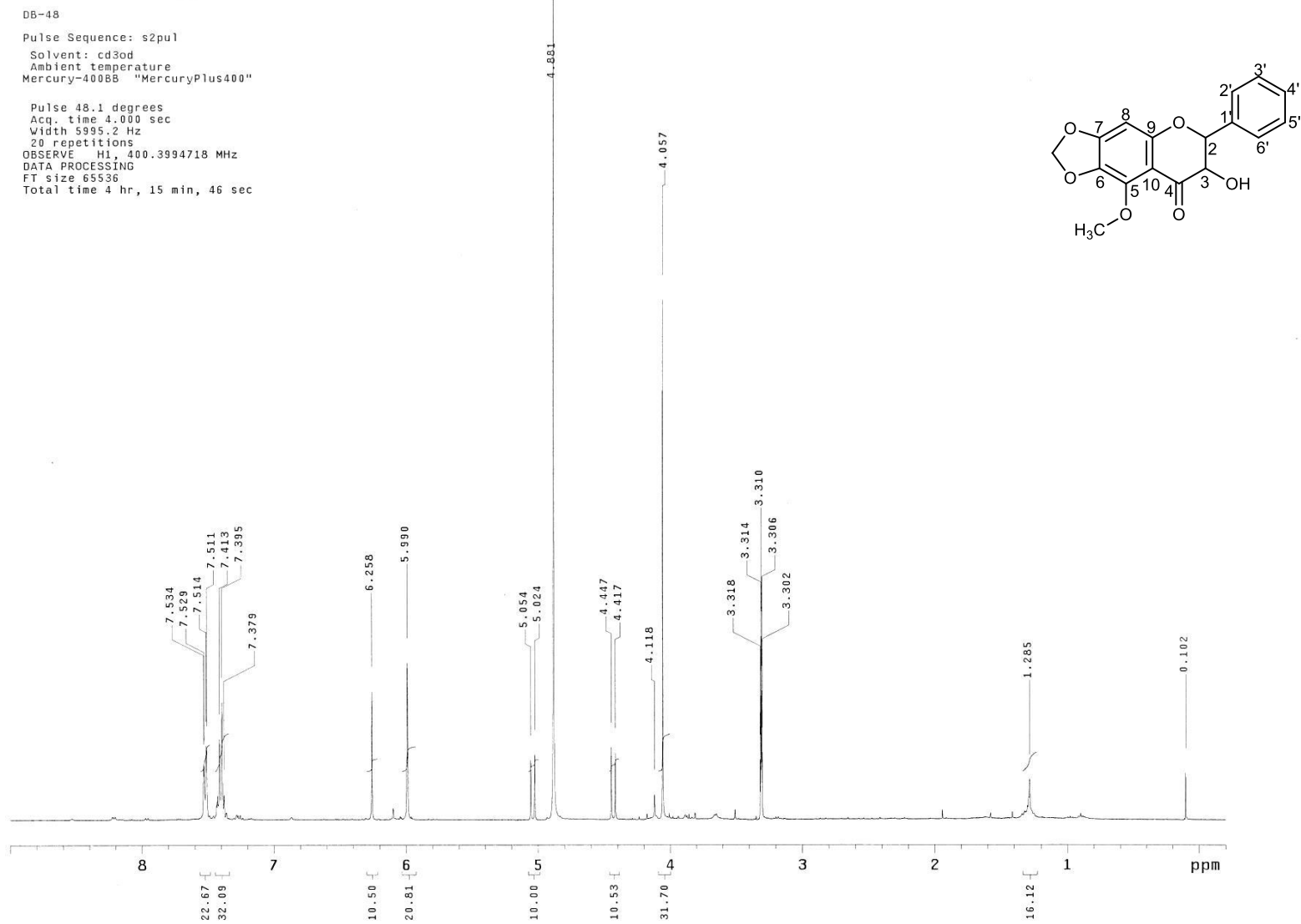
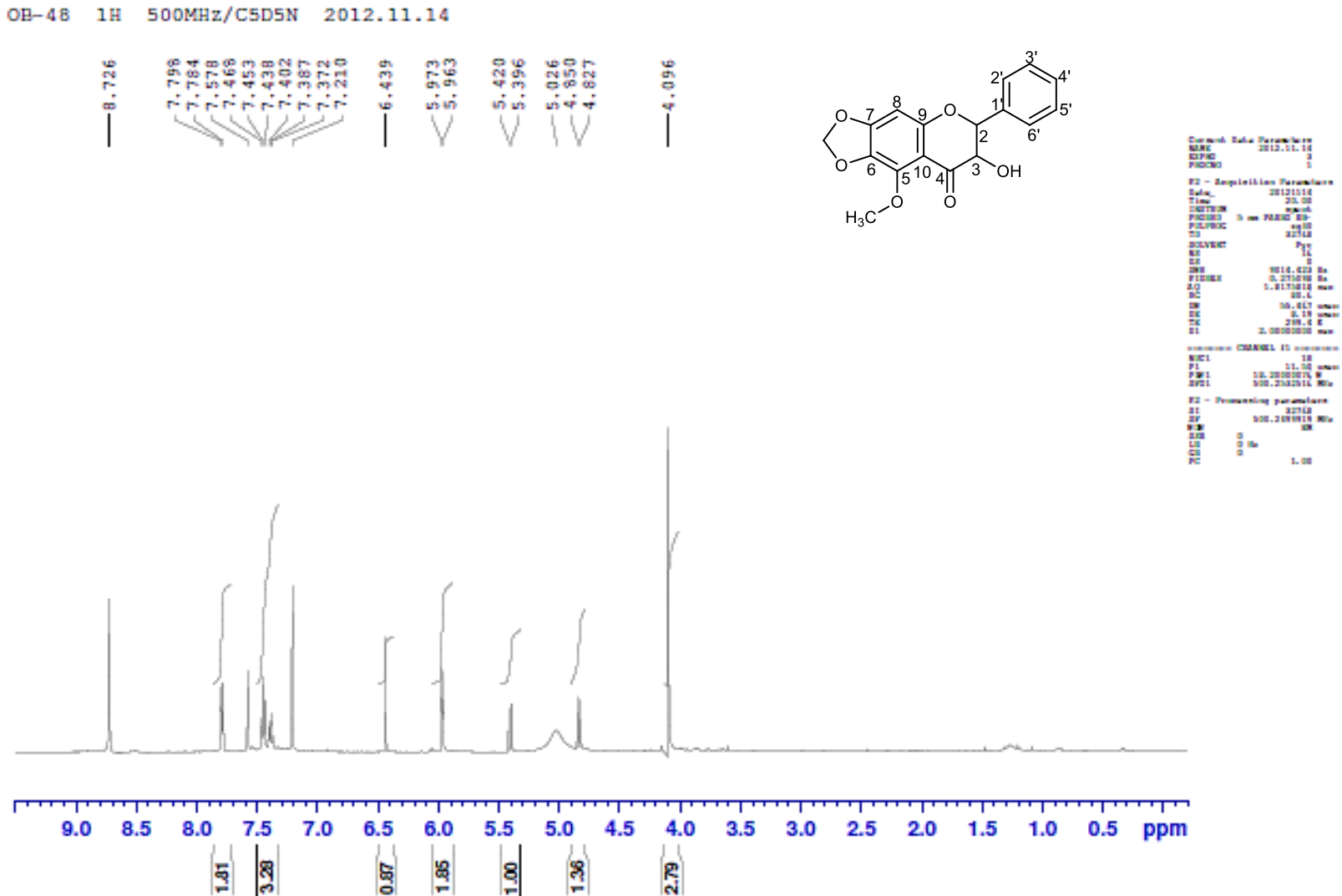
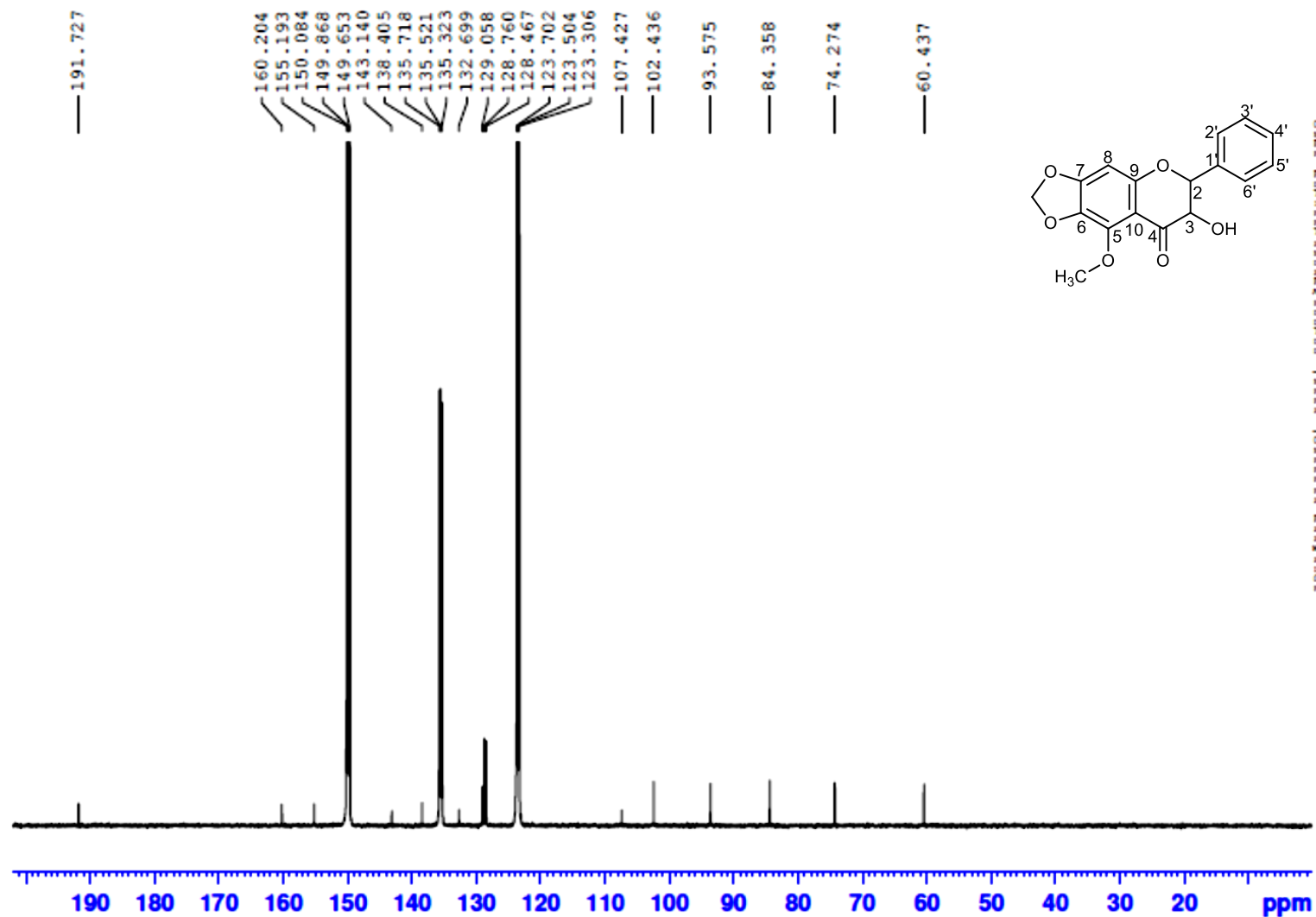


Figure S16. ^1H NMR spectrum of 3-hydroxy-5-methoxy-6,7-methylenedioxyflavanone (**2**) in pyridine- d_5



OB-48 13C 125MHz/C5D5N 2012.11.14



Current data parameters
NAME 2012.11.14
Date 2012.11.14
PROCNO 1

F2 - Acquisition parameters
Date 2012.11.14
Time 20.01
INSTRUM spect
PROBHD 5 mm HANCE
PULPROG zgpg30
TD 65536
SOLVENT py
NS 1280
DS 4
SWH 29761.964 Hz
FIDRES 0.646131 Hz
AQ 1.1010148 sec
RG 4
SM 14.800 kHz
SFE 6.60 kHz
TE 299.1 K
D1 2.00000000 sec
d11 0.03000000 sec

----- Channel f1 -----
NUC1 13C
P1 9.60 kHz
PRW1 90.00000000 Hz
SFO1 125.0017967 MHz

----- Channel f2 -----
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 kHz
PRW2 20.00000000 Hz
PWR12 0.61328000 W
PWR13 0.26669999 W
SFO2 400.2512010 MHz

F2 - processing parameters
SI 32768
SF 125.767434 MHz
WDW EM
SSB 0
LB 2.00 Hz
GB 0
PC 1.00

OB-48 dept135 500MHz/C5D5N 2012.11.14

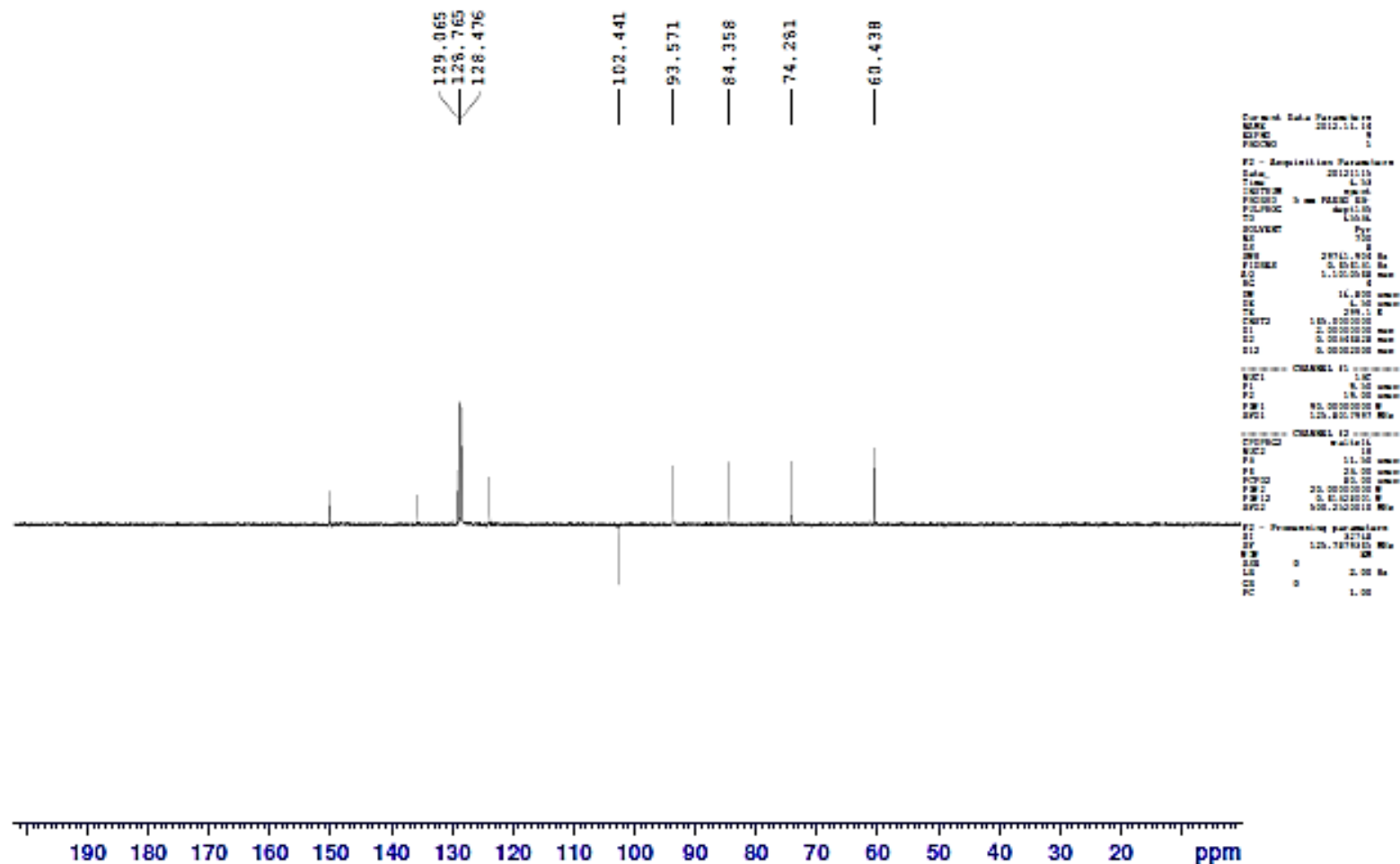


Figure S19. DEPT 45 spectrum of 3-hydroxy-5-methoxy-6,7-methylenedioxyflavanone (**2**) in pyridine-*d*₅

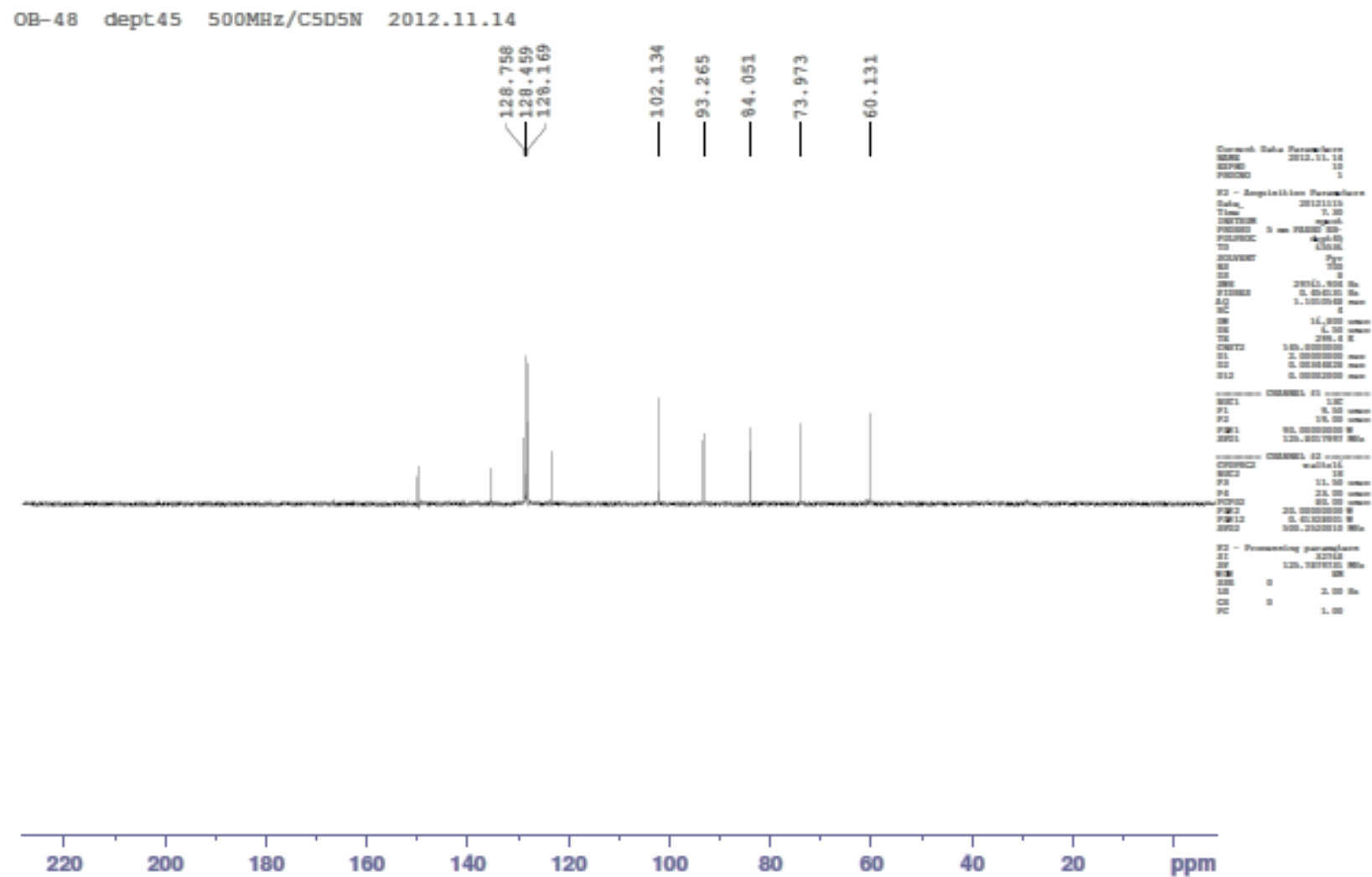


Figure S20. DEPT 90 spectrum of 3-hydroxy-5-methoxy-6,7-methylenedioxyflavanone (**2**) in pyridine-*d*₅

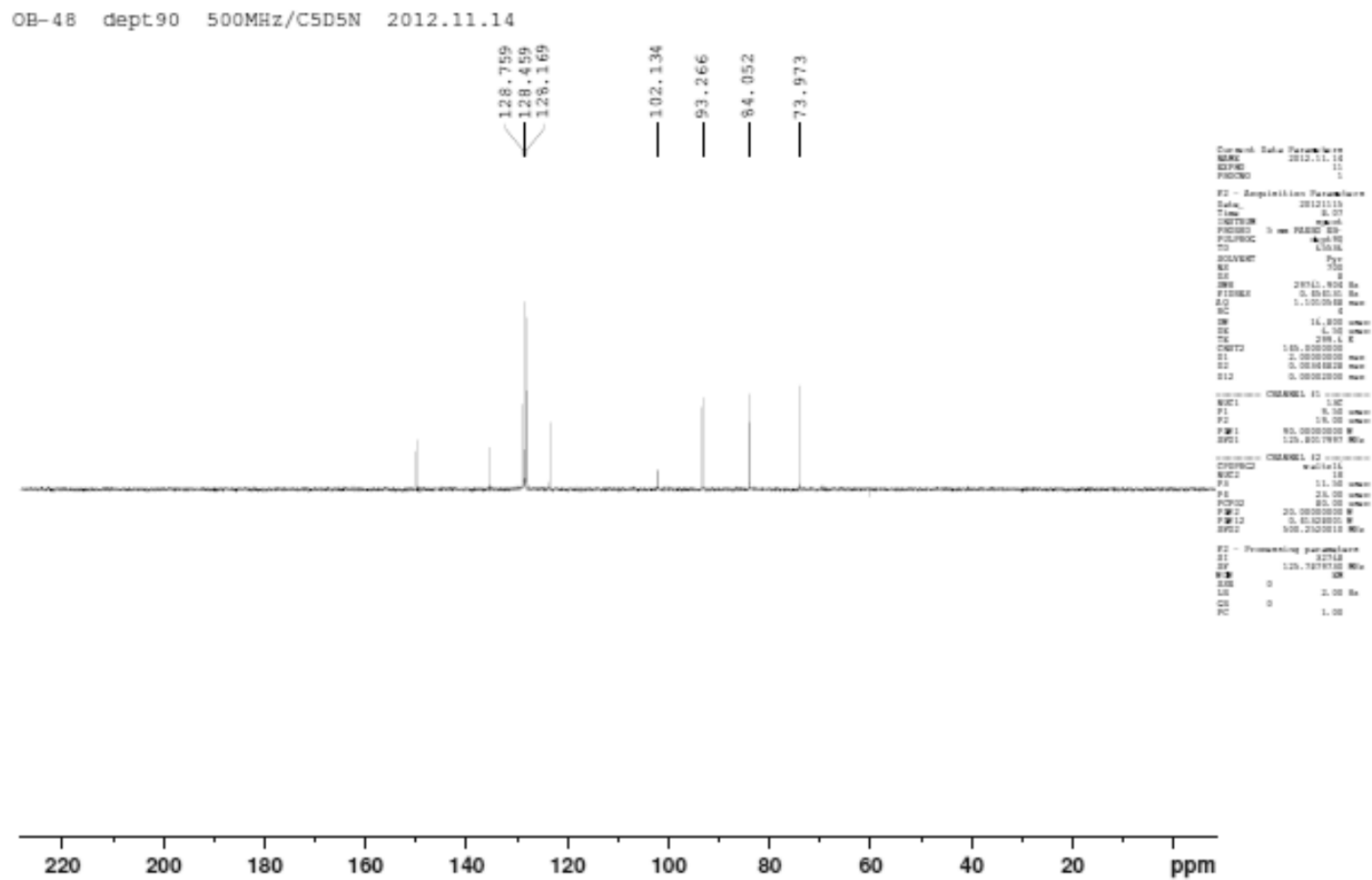


Figure S21. HSQC spectrum of 3-hydroxy-5-methoxy-6,7-methylenedioxyflavanone (**2**) in pyridine-*d*₅

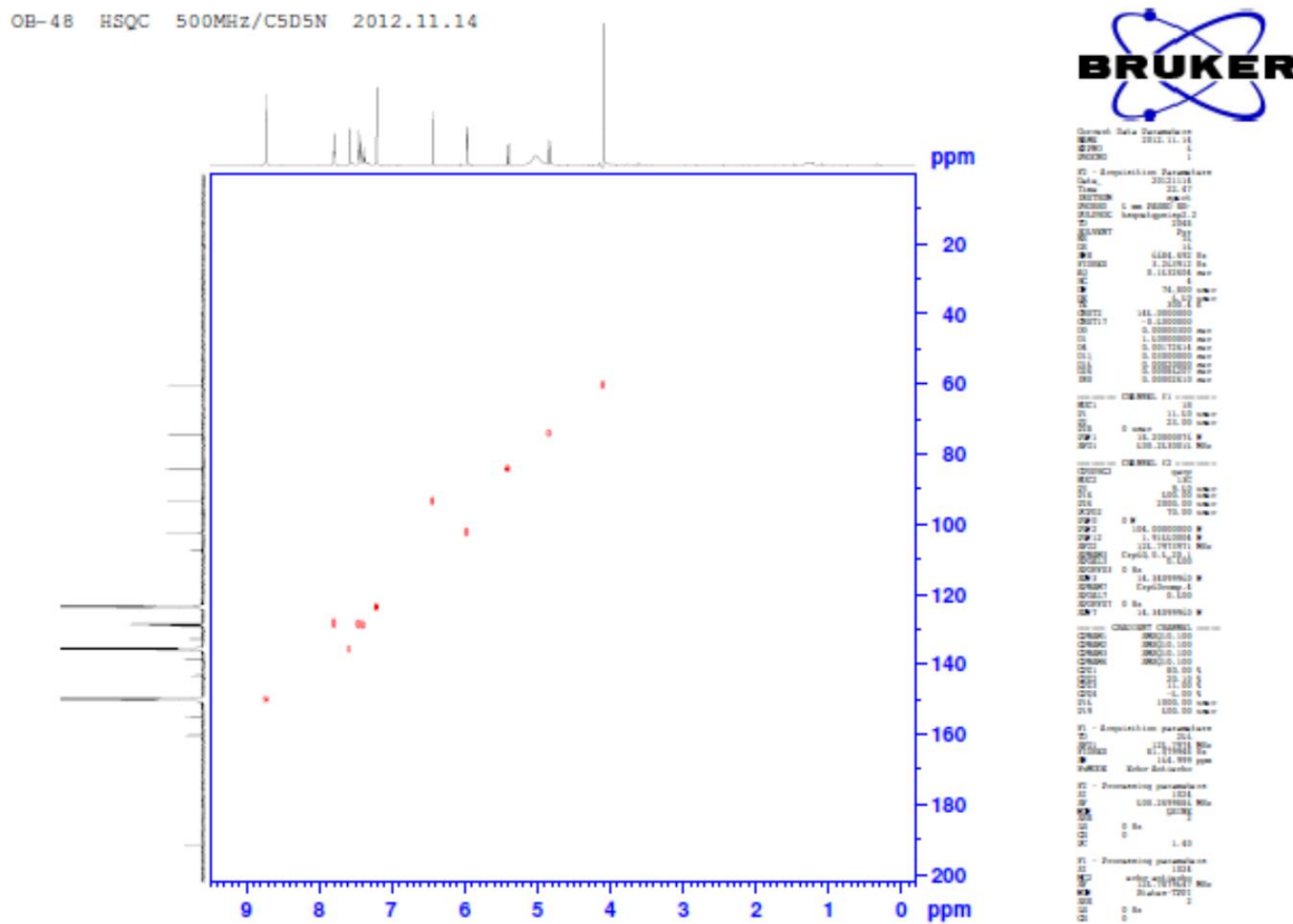
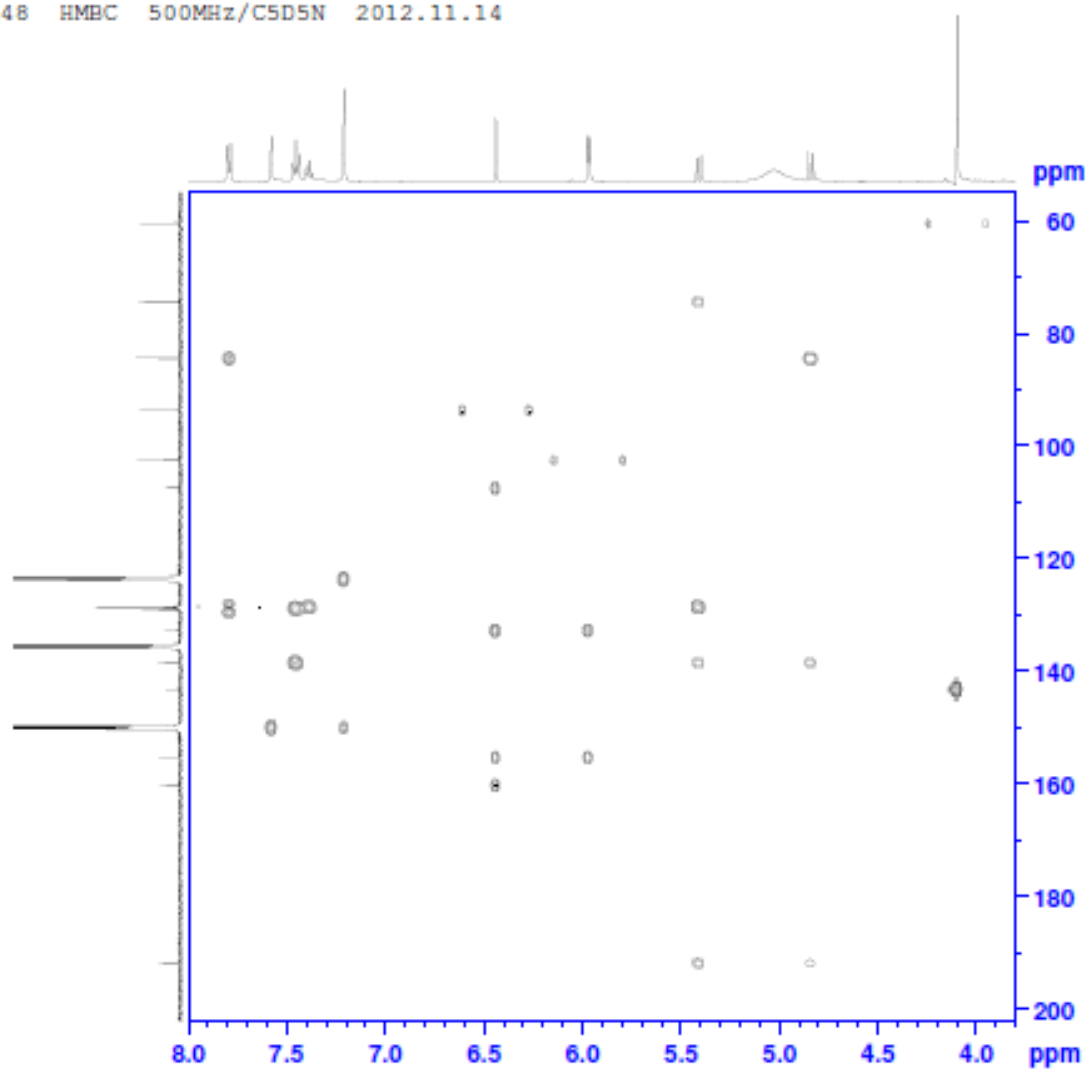


Figure S22. HMBC spectrum of 3-hydroxy-5-methoxy-6,7-methylenedioxyflavanone (**2**) in pyridine-*d*₅

OB-48 HMBC 500MHz/C5D5N 2012.11.14



Current Data Parameters
NAME: 2012.11.14
EXPNO: 7
PROCNO: 1

F2 - Acquisition Parameters
Date_: 20121113
Time: 5.33
INSTRUM: spect
PROBHD: 5 mm PABBO BB-
PULPROG: zgpg30
TD: 65536
SOLVENT: Pyr
NS: 2023
DS: 4
SWH: 6000.482 Hz
FIDRES: 3.249932 Hz
AQ: 0.1932404 sec
RG: 203
DM: 75.800 umm
DC: 6.50 umm
TE: 300.2 K
CHFT2: 160.000000
CHFT3: 0.000000
CQ: 0.0000300 umm
C1: 1.0000000 umm
C2: 0.0044828 umm
C3: 0.0430000 umm
C16: 0.0002000 umm
C80: 0.0001600 umm

CHANNEL F1
NUC1: 13C
P1: 11.50 umm
P2: 25.00 umm
PLM1: 104.0000000 W
SFO1: 100.628159 MHz

CHANNEL F2
NUC2: 1H
P3: 6.50 umm
PLM2: 104.0000000 W
SFO2: 500.1360997 MHz

GRABBER CHANNEL
CPDPRG1: ZNGD10.100
CPDPRG2: ZNGD10.100
CPDPRG3: ZNGD10.100
CPD1: 30.00 %
CPD2: 30.00 %
CPD3: 60.10 %
P16: 1000.00 umm

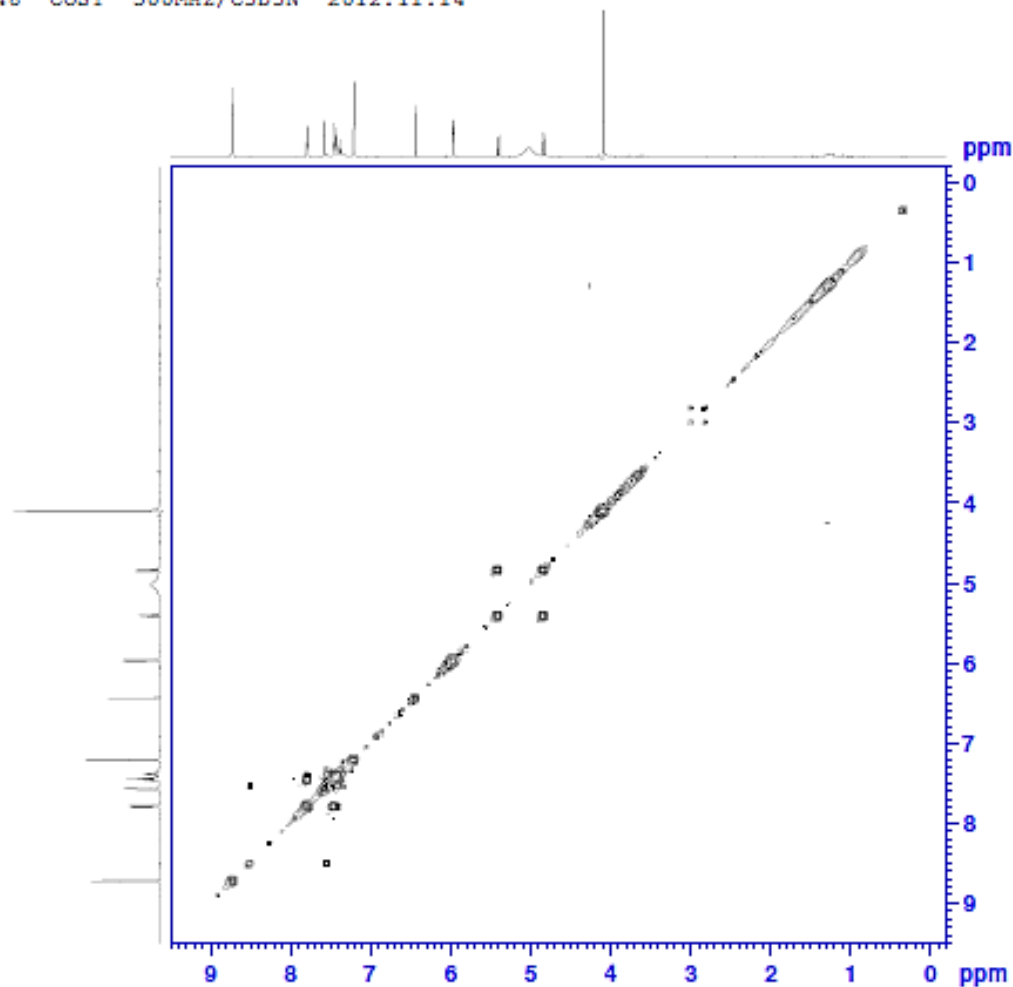
F1 - Acquisition parameters
TD: 65536
SFO1: 100.628159 MHz
FIDRES: 3.249932 Hz
DM: 75.800 ppm
PCMODE: QF

F2 - Processing parameters
SI: 32768
SF: 500.1360997 MHz
MCM: 2728
SFO2: 0
LQ: 0 Hz
CQ: 0
PC: 1.40

F1 - Processing parameters
SI: 32768
SF: 100.628159 MHz
MCM: 2728
SFO1: 0
LQ: 0 Hz
CQ: 0

Figure S23. ^1H , ^1H COSY spectrum of 3-hydroxy-5-methoxy-6,7-methylenedioxyflavanone (2) in pyridine- d_5

OB-48 COSY 500MHz/C5D5N 2012.11.14



Current Data Parameters
NAME 2012.11.14
EXPNO 5
PROCNO 1

F2 - Acquisition Parameters
Date_ 20121114
Time 21.04
INSTRUM spect
PROBHD 5 mm BBOHD 1H-
PULPROG zgpg30
TD 65536
SOLVENT py
NS 14
DS 4
SWH 6604.492 Hz
FIDRES 0.263912 Hz
AQ 0.1533404 sec
RG 64
IN 74.500 cm/sec
DE 6.50 cm/sec
TE 300.2 K
D0 0.0001300 sec
D1 1.5000000 sec
D11 0.0300000 sec
D12 0.0001000 sec
D13 0.0001400 sec
D14 0.0001000 sec
IN3 0.0001460 sec

CHANNEL f1 1H
NUC1 1H
P0 18.50 cm/sec
P1 18.50 cm/sec
P17 2500.00 cm/sec
PLN1 10.20000076 W
PLN10 3.56042004 W
SFO1 500.253015 MHz

GRABBER CHANNEL
CPDPRG1 SMSQ10.100
CPE1 10.00 %
P16 1000.00 cm/sec

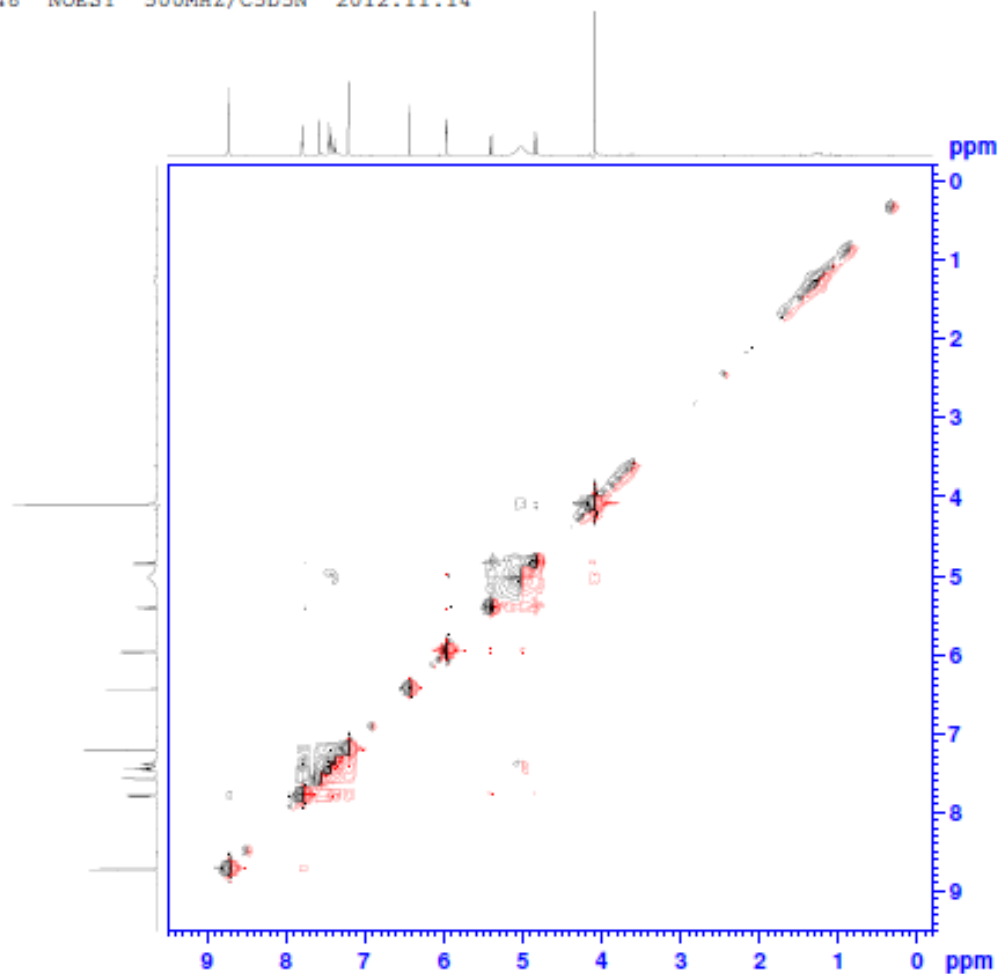
F1 - Acquisition parameters
TD 65536
SFO1 500.253 MHz
FIDRES 0.263912 Hz
SW 6604.492 Hz
PRMCK 0

F2 - Processing parameters
SI 32768
SF 500.2496903 MHz
WDW Q51MK
SSB 0
LB 0 Hz
GB 0
PC 1.40

F1 - Processing parameters
SI 32768
SF 500.2496903 MHz
WDW Q51MK
SSB 0
LB 0 Hz
GB 0

Figure S24. NOESY spectrum of 3-hydroxy-5-methoxy-6,7-methylenedioxyflavanone (**2**) in pyridine-*d*₅

OB-48 NOESY 500MHz/C5D5N 2012.11.14



```

Current Data Parameters
NAME      2012.11.14
EXPNO     1
PROCNO    1

F2 - Acquisition Parameters
Date_     20121115
Time      3.19
INSTRUM   spect
PROBHD    5 mm BBOBO BB-
PULPROG   noesypphpgp
TD        2648
SOLVENT   py
NS         16
DS         16
SWH        6684.492 Hz
FIDRES     3.263912 Hz
AQ         0.1532404 sec
RG         4
IN         74.800 usec
DE         6.50 usec
TE         299.0 K
DQ         0.0000001 sec
D1         2.00000000 sec
D2         0.40000001 sec
D11        0.00000000 sec
D12        0.00000000 sec
D14        0.00000000 sec
IN0        0.00014960 sec

----- CHANNEL f1 -----
NUC1       1H
P1         11.50 usec
P2         23.00 usec
P17        2500.00 usec
PL16       18.20000074 W
PL17       3.54000004 W
SFO1       500.2530015 MHz

----- GRADIENT CHANNEL -----
CPDPRG1    SMSQ10.100
CPS1       48.00 %
P14        1000.00 usec

F1 - Acquisition parameters
TD         264
SFO1       500.253 MHz
FIDRES     26.111298 Hz
SN         13.362 ppm
PRACQ      Stator-TFPI

F2 - Processing parameters
SI         1024
SF         500.250000 MHz
WDW        Q5TIME
SSB        2
LB         0 Hz
GB         0
PC         1.00

F1 - Processing parameters
SI         1024
MC2        Stator-TFPI
SF         500.250000 MHz
WDW        A
SSB        1
LB         0 Hz
GB         0

```

Figure S25. ESIMS⁺ spectrum of 3-hydroxy-5-methoxy-6,7-methylenedioxyflavanone (2)

