Supporting Information

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Degranulation Inhibitors from Flowers of *Coreopsis grandiflora*

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Experimental

General

Rat basophilic leukaemia (RBL-2H3) cells were obtained from JCRB Cell Bank (Osaka, Japan) and maintained under the culture conditions recommended by the cell bank. Dulbecco's modified Eagle's medium (DMEM), trypsin-ethylenediamine tetraacetic acid (trypsin-EDTA), mouse anti-dinitrophenol (anti-DNP) monoclonal IgE, DNP-conjugated human serum albumin (DNP-HSA) and Triton X-100 were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Dimethyl sulfoxide (DMSO), glycine, glucose, sodium chloride (NaCl), calcium chloride ($CaCl_2$), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (PNAG), oxatomide, *n*-hexane, EtOAc, 1-BuOH, AcOH, acetone, MeOH and toluene were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) was obtained from Dojindo Laboratories (Kumamoto, Japan). Fetal bovine serum (FBS) (Lot. 42F9155K) was obtained from Gibco (Waltham, MA, USA). Penicillin-streptomycin mixed solution, potassium chloride (KCl) and bovine serum albumin (BSA) were obtained from Nacalai Tesque (Kyoto, Japan). Ninety-six-well plates (167008, Nunc) were obtained from Thermo Fisher Scientific K.K. (Tokyo, Japan). DIAION HP20 (Mitsubishi Chemical Corporation, Tokyo, Japan), Wakogel C-200 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), TOYOPEARL HW-40F (Tosoh Corporation, Tokyo, Japan) and Inertsil Ph-3 (GL Sciences, Tokyo, Japan) were used for column chromatography. The preparative HPLC equipment used was SHIMADZU products (Kyoto, Japan) consisting of a pump (LC-10AD), a UV-Vis detector (SPD-10AV), a column oven (CTO-6A) and chromatopac (C-R7A plus). ^{1}H-NMR, ^{13}C-NMR, HSQC and HMBC spectra were obtained on a Varian NMR System 600 MHz instrument with CD_3OD (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The values of chemical shifts are expressed in ppm, and each coupling constant (*J*) is expressed in Hz. Electron spray ionization (ESI) high-resolution mass spectra were recorded on a Bruker Daltonics MicrOTOF II instrument using direct sample injection.

Isolation of compounds 1-4 from the extract of petals from Coreopsis grandiflora

Petals from *Coreopsis grandiflora* (1.55 kg, fr. wt) were obtained in Prefectural University of Hiroshima (Shobara, Japan) on May 25, 2018. For extraction, the collected

petals were mixed with 9.3 L of MeOH-H_2O (70/30, *v/v*) at room temperature for 1 week. After filtration, the petal extract was concentrated to an approximate volume of 700 ml. The concentrated extract was partitioned with *n*-hexane (700 ml, twice), EtOAc (700 ml, twice) and water-saturated 1-BuOH (350 ml, twice) in that order. The EtOAc layer (15.8 g), which showed degranulation inhibitory activity, was applied to a DIAION HP20 column (ϕ 7.0 x 32.0 cm) and eluted with a stepwise MeOH-H_2O-CH_3COOH gradient (fraction size 200 ml; 20/79/1, 40/59/1, 60/39/1, 80/19/1, 99/0/1 each 2 L, *v/v/v*) and with 3 L of acetone, and fractions eluted with MeOH-H_2O-CH_3COOH (20/79/1, *v/v/v*) (fraction A) and fractions eluted with MeOH-H_2O-CH_3COOH (99/0/1, *v/v/v*) (fraction B) showed degranulation inhibitory activities. Fraction A (8.3 g) was chromatographed on Wakogel C-200 (ϕ 7.0 x 40.1 cm) and eluted with a stepwise hexane-acetone gradient (70/30: 2.5 L, 60/40: 2.5 L, 50/50: 3.0 L, 0/100: 4.5 L, *v/v*) to isolate an active compound eluted with hexane-acetone (60/40, *v/v*) (compound **1**, 223.4 mg) and to obtain active fractions eluted with hexane-acetone (50/50, *v/v*) (fraction C). Fraction C (731.0 mg) was further purified by TOYOPEARL HW-40F (ϕ 2.5 x 97.2 cm) with MeOH-H_2O-CH_3COOH (80/19.8/0.2, *v/v/v*) to isolate two active compounds (compound **2**, 407.3 mg, and compound **3**, 49.6 mg). On the other hand, fraction B (602.4 mg) was chromatographed on Wakogel C-200 (ϕ 2.0 x 39.4 cm) and eluted with a stepwise hexane-acetone gradient (80/20: 180 ml, 70/30: 180 ml, 60/40: 180 ml, 50/50: 360 ml, *v/v*). The eluted fractions in hexane-acetone (60/40, *v/v*) (fraction D) showed significant activity. Moreover, a part (82.7 mg) of fraction D was dissolved with MeOH-H_2O-CH_3COOH (55/44/1, *v/v/v*) and then subjected to preparative HPLC. Preparative HPLC of an active compound was carried out on an Inertsil Ph-3 column (ϕ 10 x 250 mm, 5 μm). An isocratic system of MeOH-H_2O-CH_3COOH (55/44/1, *v/v/v*) was used as the mobile phase with a flow rate of 3.3 ml/min. The detection wavelength was 280 nm, and the injection volume was 180 μl when an active compound was purified, and then an active compound (compound **4**, 40.3 mg) was isolated.

Compound **1** (lanceoletin); Yellow powder. HRMS: *m/z* 301.0716 $[M-H]^-$ (calcd. for $C_{16}H_{13}O_6^-$, 301.0718). The ^{1}H-NMR data of compound **1** are recorded and assigned in Table S1. These ^{1}H-NMR data were consistent with those of lanceoletin in another report [1].

Compound **2** (leptosidin); Deep yellow powder. HRMS: *m/z* 299.0567 $[M-H]^-$ (calcd. for $C_{16}H_{11}O_6^-$, 299.0561). The ^{1}H-NMR data of compound **2** are recorded and assigned in Table S2.

Leptosidin was isolated from flower parts of *Coreopsis grandiflora* prior to this study; Deep yellow powder. HRMS: *m/z* 299.0566 $[M-H]^-$ (calcd. for $C_{16}H_{11}O_6^-$, 299.0561). The ^1H and ^{13}C-NMR data of the isolated compound are recorded and assigned in Table S2. The chemical structure was determined by ^1H-NMR, ^{13}C-NMR, HSQC (Figures S4-S6) and HRMS spectra. The geometry of carbon-carbon double bond at the C-2 and C-10 positions was assigned as *Z* judging from the chemical shift value at H-10 proton of ^1H-NMR spectrum [2,3] and that at C-10 carbon of ^{13}C-NMR spectrum [4]. Compound **2** isolated from the petals of flowers was identified as leptosidin by ^1H-NMR and HRMS and by comparison of its spectroscopic data with those of leptosidin.

Compound **3** (okanin); Yellow powder. HRMS: *m/z* 287.0559 $[M-H]^-$ (calcd. for $C_{15}H_{11}O_6^-$, 287.0561). The ^1H and ^{13}C-NMR data of compound **3** are recorded and assigned in Table S3. The chemical structure was determined by ^1H-NMR, ^{13}C-NMR, HSQC and HMBC (Figures S7-S10).

Compound **4** (4-methoxylanceoletin); Yellow powder. HRMS: *m/z* 315.0885 $[M-H]^-$ (calcd. for $C_{17}H_{15}O_6^-$, 315.0874). The ^1H and ^{13}C-NMR data of compound **4** are recorded and assigned in Table S4. The chemical structure was determined by ^1H-NMR, ^{13}C-NMR, HSQC and HMBC (Figures S11-S14).

Antigen-stimulated degranulation assay

The inhibitory activities of various compounds against IgE-stimulated degranulation from RBL-2H3 cells were evaluated by modifying the method of Watanabe *et al* [5]. RBL-2H3 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Then RBL-2H3 cells from stock cultures were suspended in the medium and plated at 5.0 x 10^4 cells/200 μl/well in 96-well plates and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. After 24 h, the cells were incubated in 100 μl of growth medium containing 50 ng/ml of mouse monoclonal anti-DNP IgE for 2 h. The incubated cells were washed with modified Tyrode (MT) buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2 \cdot 6H_2O$, 5.6 mM glucose, 20 mM HEPES, 0.1% BSA, pH 7.3) before 90 μl of each of the test compounds or oxatomide was added. The test compounds and oxatomide were dissolved in DMSO and diluted with MT buffer to obtain a final DMSO concentration of 0.25%. After 20-min incubation, 10 μl of DNP-labeled human serum albumin (final concentration: 50 ng/ml) was

added to the cells, and the cultures were incubated for 1 h. The supernatants were collected, and the cells were lysed with 100 µl of MT buffer containing 0.1% Triton X-100. The β-hexosaminidase activities of the supernatants and cell lysates were measured using the method reported by Demo *et al* [6]. Each 20 µl of aliquot of the supernatant or cell lysate was mixed with a 40 µl volume of 3.3 mM PNAG in 100 mM citrate buffer (pH 4.5), and the mixture was incubated in a 96-well plate at 37 °C for 90 min. Each reaction was terminated by adding 40 µl of 2 M glycine buffer (pH 10.4), and the absorbance of each well at 405 nm was measured using a microplate reader (Varioskan FC from Thermo Fisher Scientific, Waltham, MA, USA).

Degranulation ratio (%) = [St-Sb / { (St-Sb) + C }] x 100.

In this equation, St, Sb and C express the absorbance of sample-treated (St), sample-blank (Sb) and cell lysate (C) with the stimulant only, respectively.

Ca^{2+} ionophore-stimulated degranulation assay

The inhibitory activities of oxatomide and the isolated compounds against calcium ionophore-stimulated degranulation from RBL-2H3 cells were evaluated according to a previously published method [7]. RBL-2H3 cells were cultured at 5.0 x 10^4 cells/200 µl/well in a 96-well plate for 24 h at 37 °C under a humidified atmosphere with 5% CO_2. The cells were washed with MT buffer before the addition of 90 µl of oxatomide or test compounds as described in the previous section. After 20-min incubation, 10 µl of calcium ionophore A23187 (final concentration: 1 µM) was added to the cells and the cultures were incubated for 1 h. The supernatants were collected, and the cells were lysed with MT buffer containing 0.1% Triton X-100. The degranulation assay was performed as described in the previous section.

Results and discussion

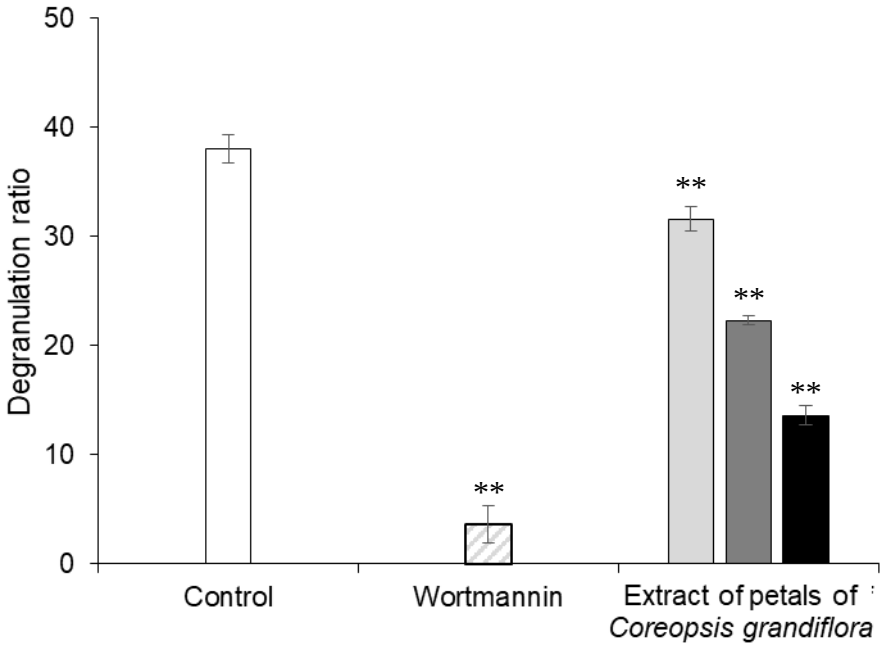
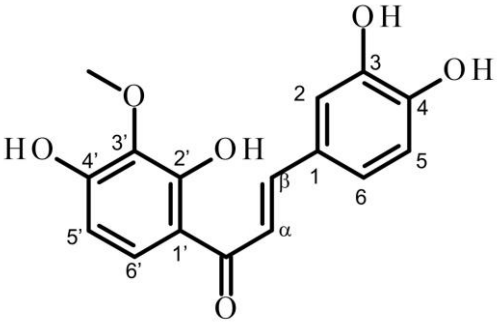


Figure S1: Inhibitory effects of extract of petals of *Coreopsis grandiflora* on antigen-induced degranulation in RBL-2H3 cells.

Wortmannin (2.5 μM) was used as positive control. Anti-dinitrophenyl (DNP)-immunoglobulin E-sensitized RBL-2H3 cells were incubated with 2.5 μM of wortmannin or 20, 40 and 60 μg/mL of extract of *Chrysanthemum grandiflora* and stimulated with DNP-human serum albumin. All data represent means ± SD of triplicate cultures. $^{**}p < 0.01$ (Dunnett's test) as compared with the control.

Table S1: NMR data of compound **1** (600 MHz for ^{1}H) in CD_3OD.

position	δ_H
1	
2	7.18 (1H, d, *J* = 2.4 Hz)
3	
4	
5	6.82 (1H, d, *J* = 7.8 Hz)
6	7.12 (1H, dd, *J* = 2.4, 7.8 Hz)
1'	
2'	
3'	
4'	
5'	6.48 (1H, d, *J* = 9.6 Hz)
6'	7.76 (1H, d, *J* = 9.6 Hz)
H-α	7.54 (1H, d, *J* = 15.6 Hz)
H-β	7.74 (1H, d, *J* = 15.6 Hz)
3'-OMe	3.85 (3H, s)



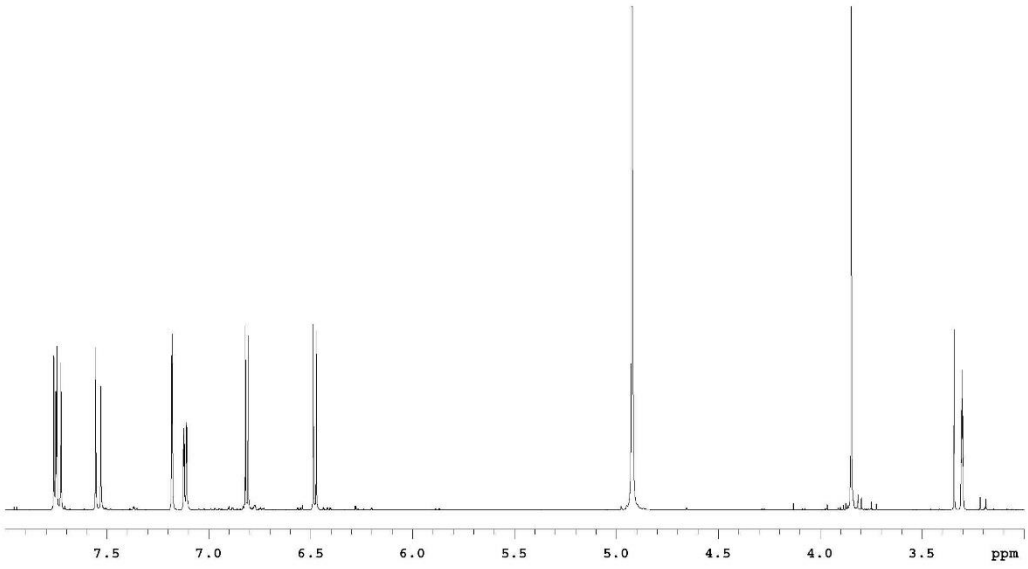
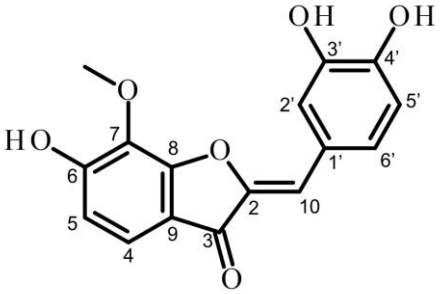


Figure S2: ^{1}H-NMR spectrum of compound **1** (lanceoletin).

Table S2: NMR data of compound **2** (600 MHz for ^{1}H) in CD_3OD.

position	δ_H
1	
2	
3	
4	7.35 (1H, d, *J* = 8.4 Hz)
5	6.74 (1H, d, *J* = 8.4 Hz)
6	
7	
8	
9	
10	6.72 (1H, s)
1'	
2'	7.48 (1H, d, *J* = 2.4 Hz)
3'	
4'	
5'	6.85 (1H, d, *J* = 7.8 Hz)
6'	7.27 (1H, dd, *J* = 7.8, 2.4 Hz)
7-OMe	4.13 (3H, s)



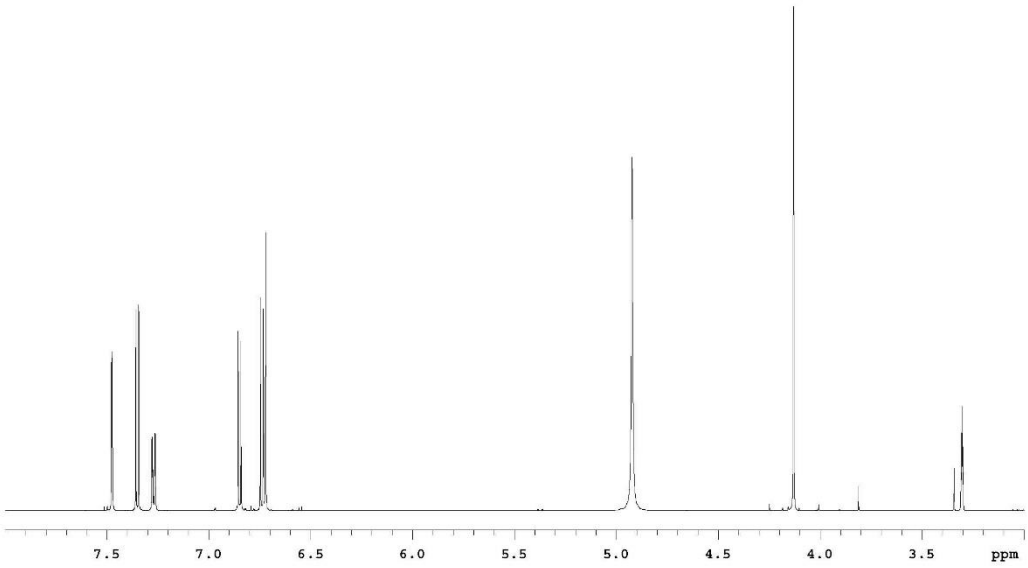
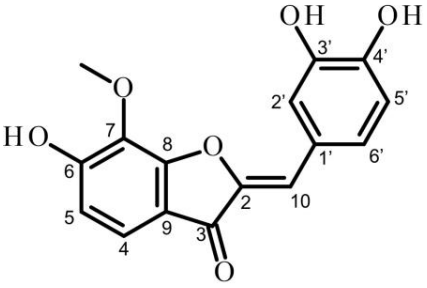


Figure S3: ^{1}H-NMR spectrum of compound **2** (leptosidin).

Table S3: NMR data of the compound isolated from flower parts of *Coreopsis grandiflora* (600 MHz for ^{1}H and 150 MHz for ^{13}C) in CD_3OD.

position	δ_H	δ_C
1		
2		146.0
3		183.1
4	7.35 (1H, d, *J* = 7.8 Hz)	119.3
5	6.74 (1H, d, *J* = 7.8 Hz)	113.1
6		158.1
7		132.3
8		158.4
9		115.2
10	6.72 (1H, s)	113.7
1'		145.4
2'	7.48 (1H, d, *J* = 1.8 Hz)	117.5
3'		123.9
4'		148.2
5'	6.85 (1H, d, *J* = 8.4 Hz)	115.3
6'	7.27 (1H, dd, *J* = 1.8, 4.8 Hz)	125.0
7-OMe	4.13 (3H, s)	60.1



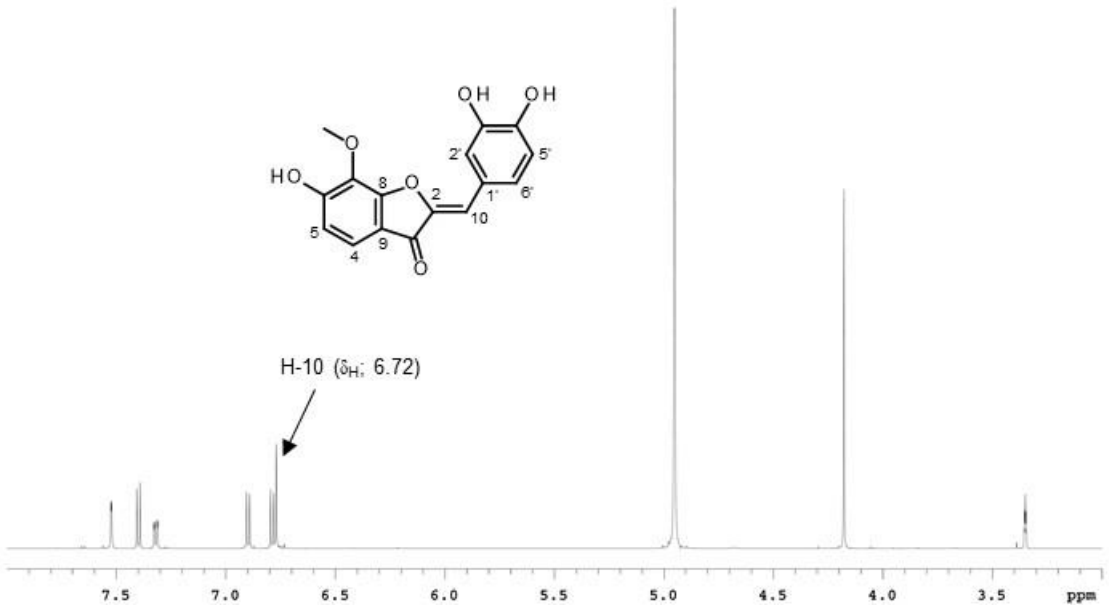


Figure S4: ^{1}H-NMR spectrum of leptosidin isolated from flower parts of *Coreopsis grandiflora*.

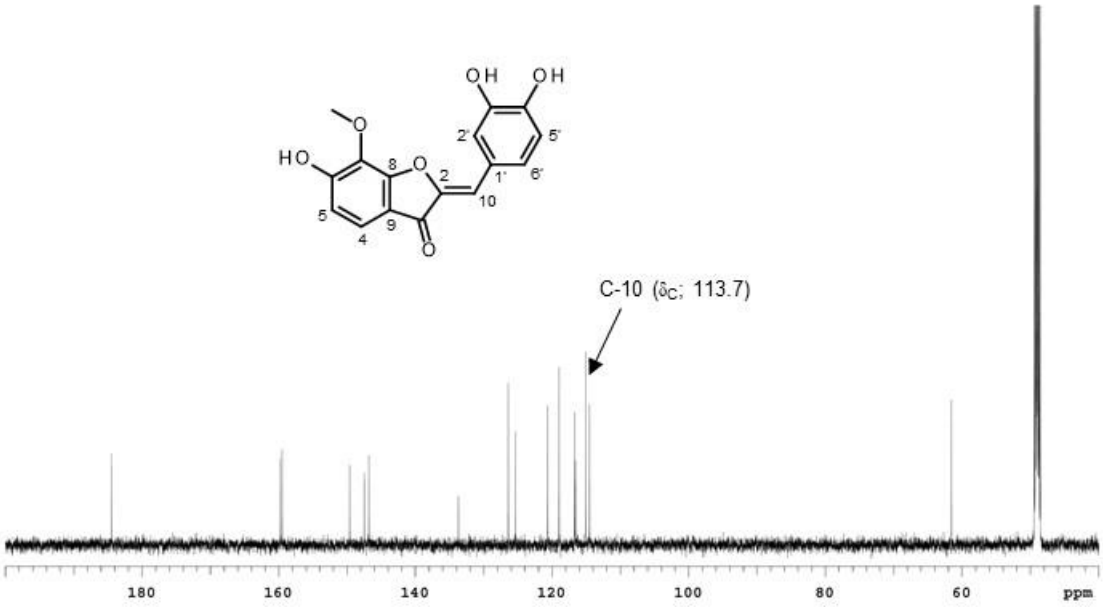


Figure S5: ^{13}C-NMR spectrum of leptosidin isolated from flower parts of *Coreopsis grandiflora*.

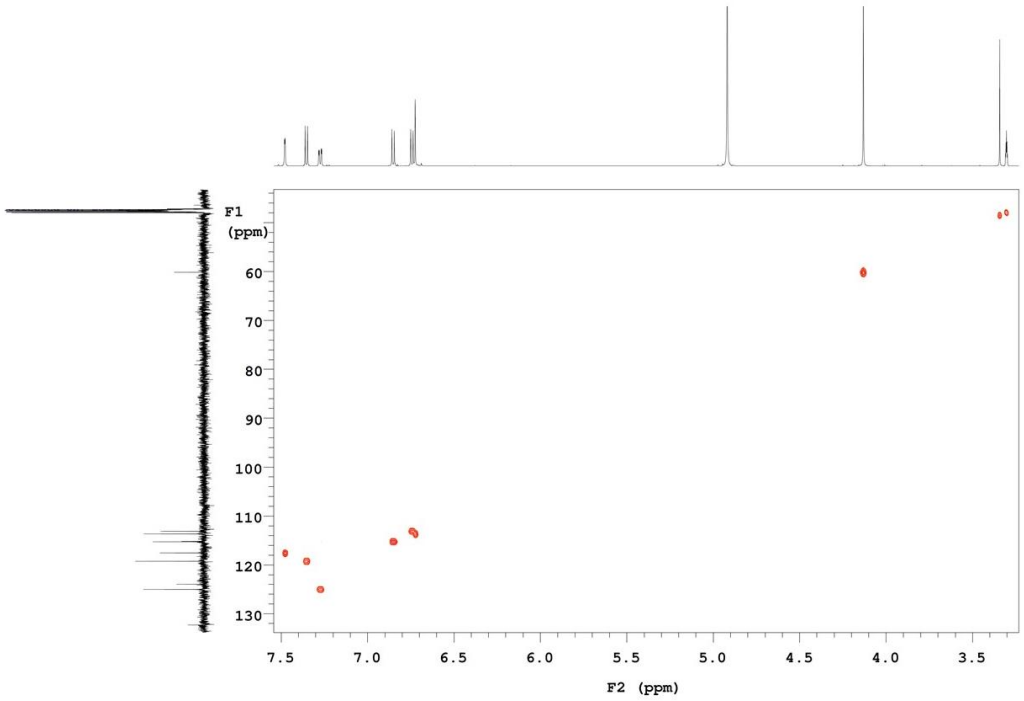
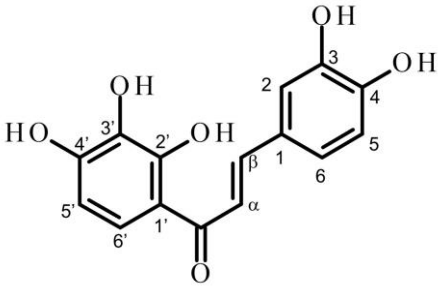


Figure S6: HSQC spectrum of leptosidin isolated from flower parts of *Coreopsis grandiflora*.

Table S4: NMR data of compound **3** (600 MHz for ^{1}H and 150 MHz for ^{13}C) in CD_3OD.

position	δ_H	δ_C
1		127.0
2	7.18 (1H, d, *J* = 1.8 Hz)	114.3
3		145.4
4		148.5
5	6.81 (1H, d, *J* = 7.8 Hz)	115.2
6	7.11 (1H, dd, *J* = 7.8, 1.8 Hz)	122.2
1'		132.3
2'		153.0 or 151.9
3'		113.7
4'		153.0 or 151.9
5'	6.46 (1H, d, *J* = 9.6 Hz)	107.1
6'	7.53 (1H, d, *J* = 9.6 Hz)	121.7
H-α	7.54 (1H, d, *J* = 15.0 Hz)	116.9
H-β	7.72 (1H, d, *J* = 15.0 Hz)	144.6
C=O		192.6



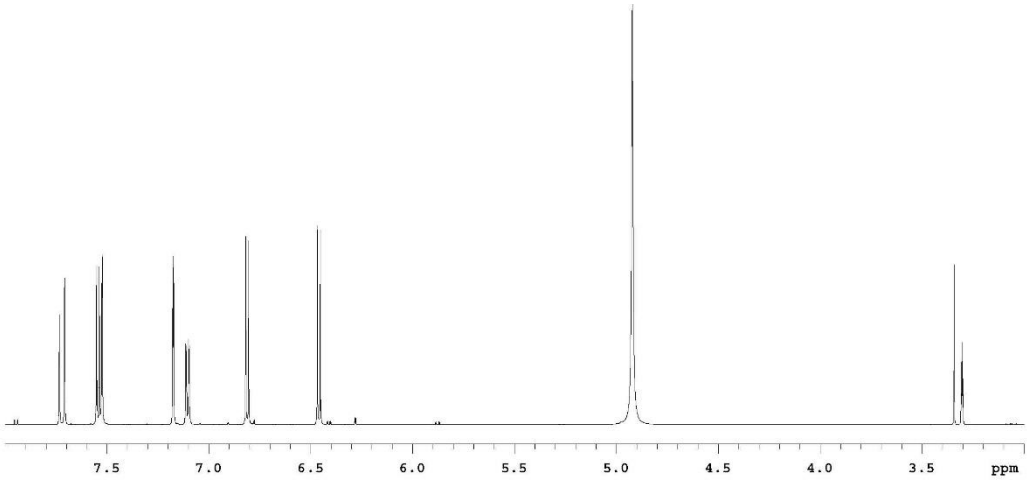


Figure S7: ^{1}H-NMR spectrum of compound **3** (okanin).

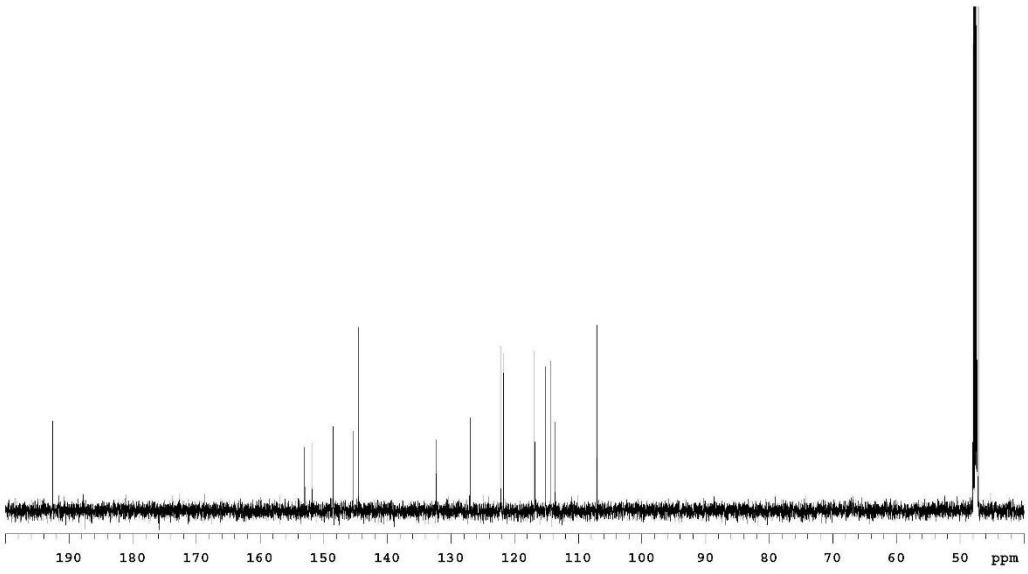


Figure S8: ^{13}C-NMR spectrum of compound **3** (okanin).

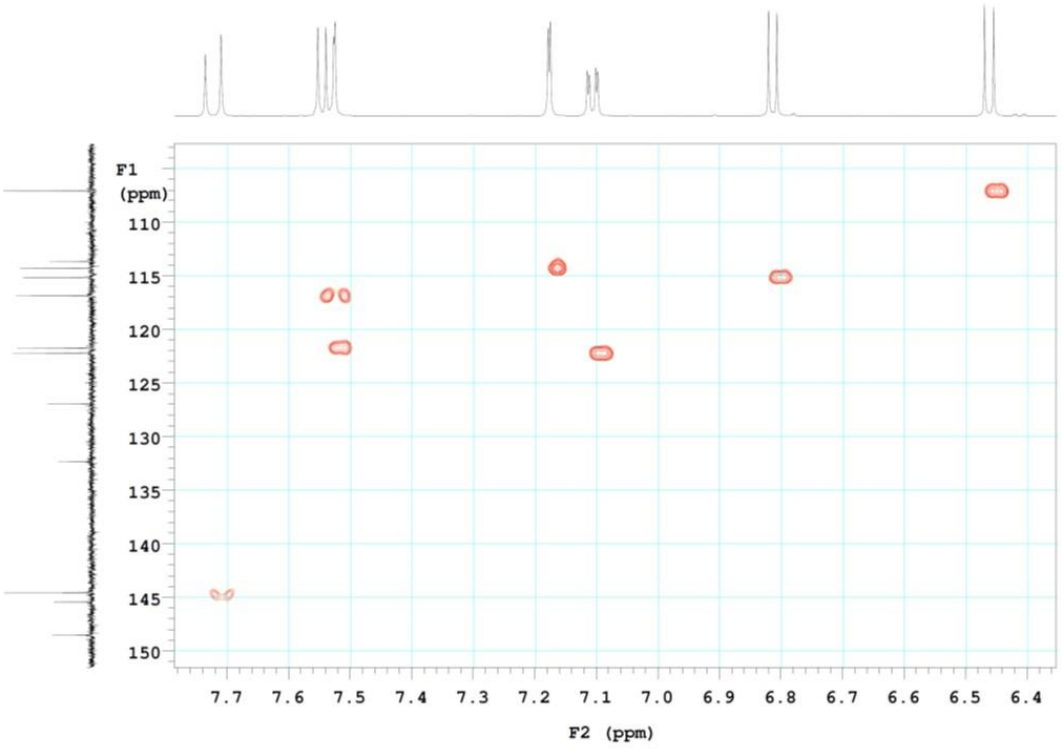


Figure S9: HSQC spectrum of compound **3** (okanin).

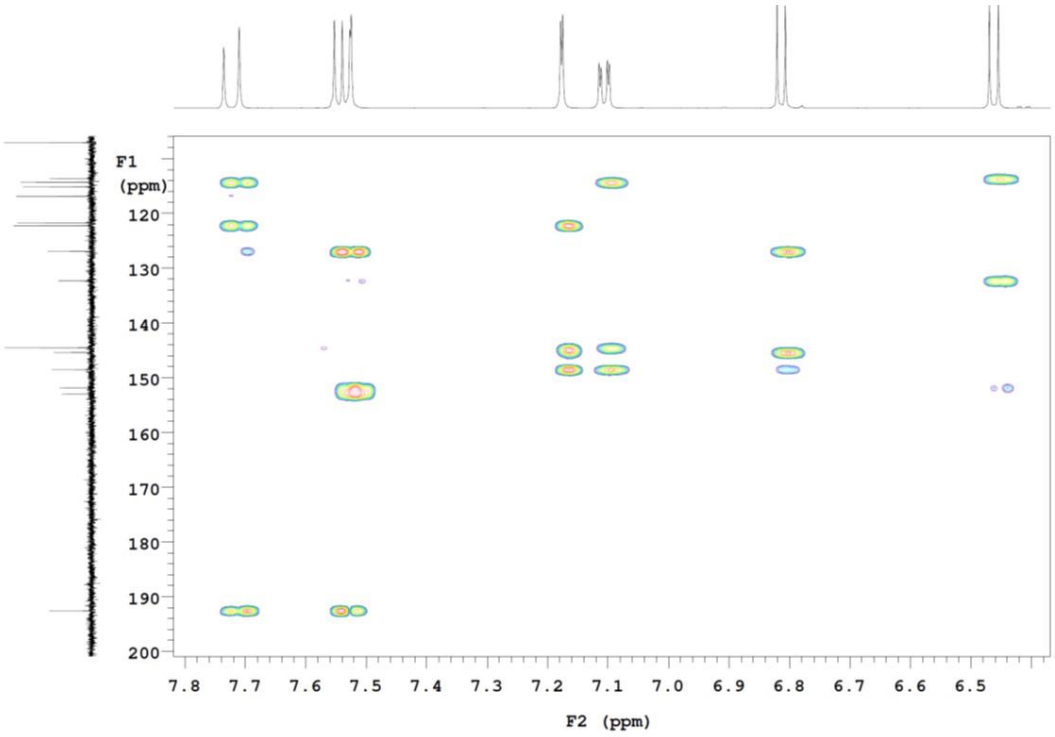
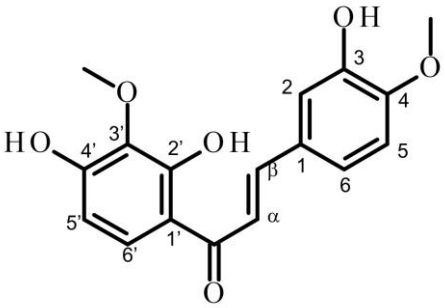


Figure S10: HMBC spectrum of compound **3** (okanin).

Table S5: NMR data of compound **4** (600 MHz for ^{1}H and 150 MHz for ^{13}C) in CD_3OD.

position	δ_H	δ_C
1		128.0
2	7.23 (1H, d, *J* = 1.8 Hz)	113.8
3		146.6
4		150.4
5	6.97 (1H, d, *J* = 8.4 Hz)	111.0
6	7.20 (1H, dd, *J* = 8.4, 1.8 Hz)	122.2
1'		114.0
2'		158.4 or 157.1
3'		134.8
4'		158.4 or 157.1
5'	6.48 (1H, d, *J* = 9.0 Hz)	107.6
6'	7.76 (1H, d, *J* = 9.0 Hz)	126.4
H-α	7.59 (1H, d, *J* = 15.6 Hz)	117.7
H-β	7.75 (1H, d, *J* = 15.6 Hz)	144.4
3'-OMe	3.90 (3H, s)	54.9
4-OMe	3.85 (3H, s)	59.4
C=O		192.5



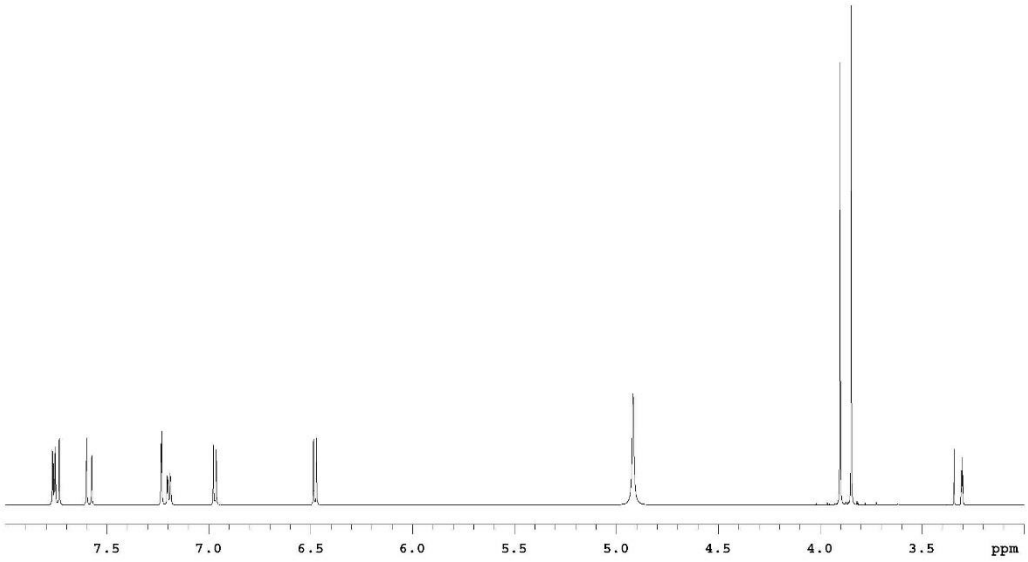


Figure S11: ^{1}H-NMR spectrum of compound **4** (4-methoxylanceoletin).

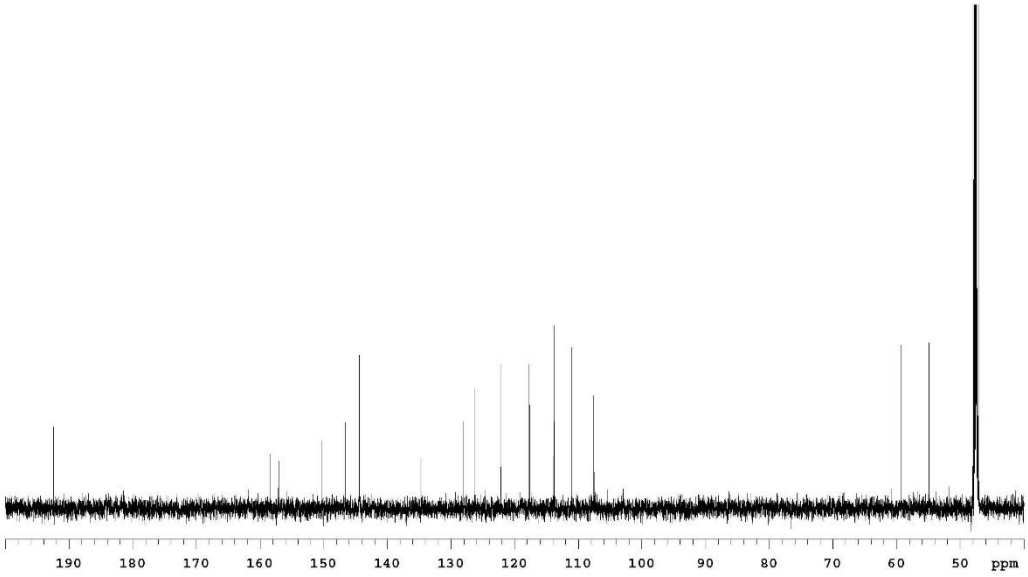


Figure S12: ^{13}C-NMR spectrum of compound **4** (4-methoxylanceoletin).

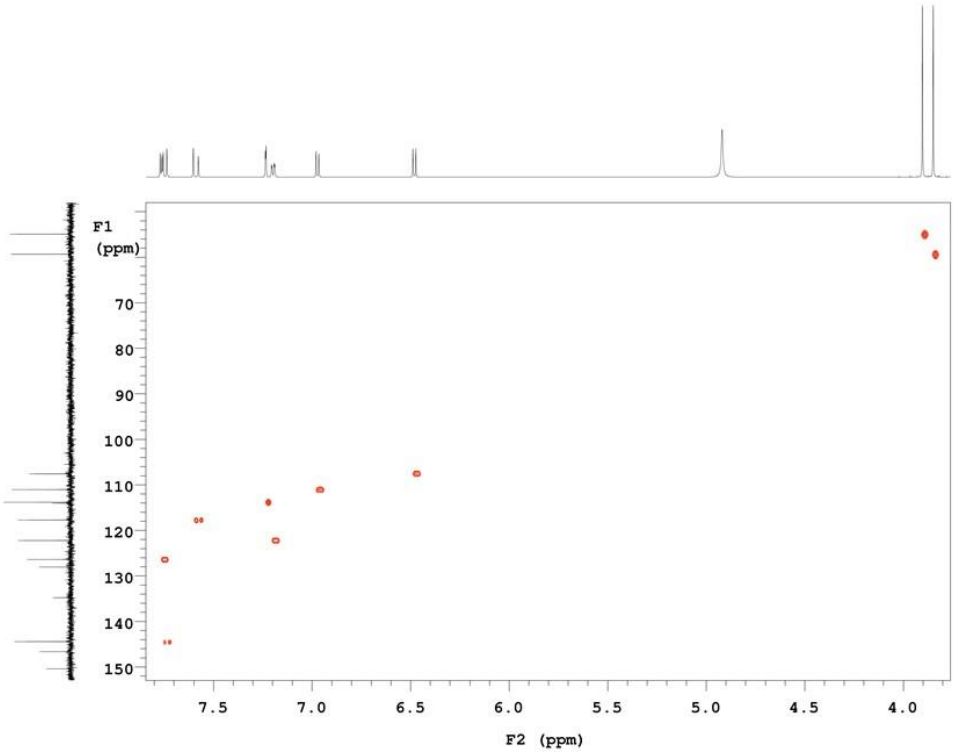


Figure S13: HSQC spectrum of compound **4** (4-methoxylanceoletin).

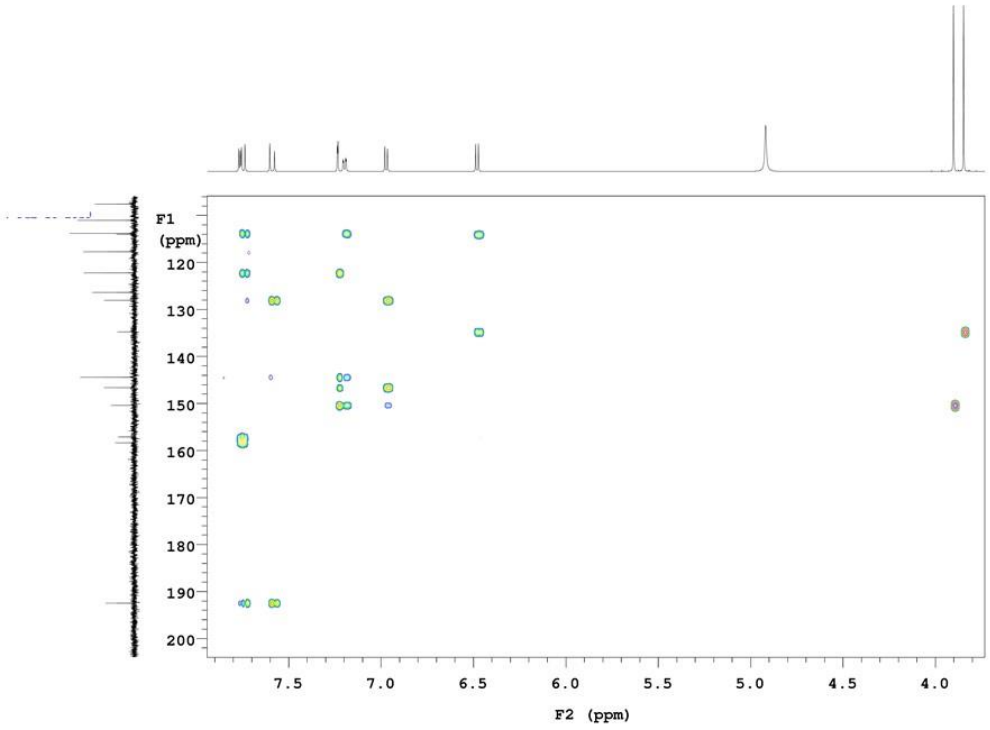


Figure S14: HMBC spectrum of compound **4** (4-methoxylanceoletin).

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