Estrogen Receptor (ER) Subtype Selectivity Identifies 8-Prenylapigenin as an ERβ Agonist from *Glycyrrhiza inflata* and Highlights the Importance of Chemical and Biological Authentication

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SUPPORTING INFORMATION

Note: The raw and annotated NMR data, as well as the ^{1}H iterative full spin analysis (HiFSA) of 8-prenylapigenin (syn, licoflavone C), 4'-*O*-methylbavachalcone (= 4'-*O*-methylroussochalcone B) and its isomer (bavachinin), abyssinone II, as well as licochalcone C are made freely available via the Harvard Dataverse at doi:10.7910/DVN/JZOL2U

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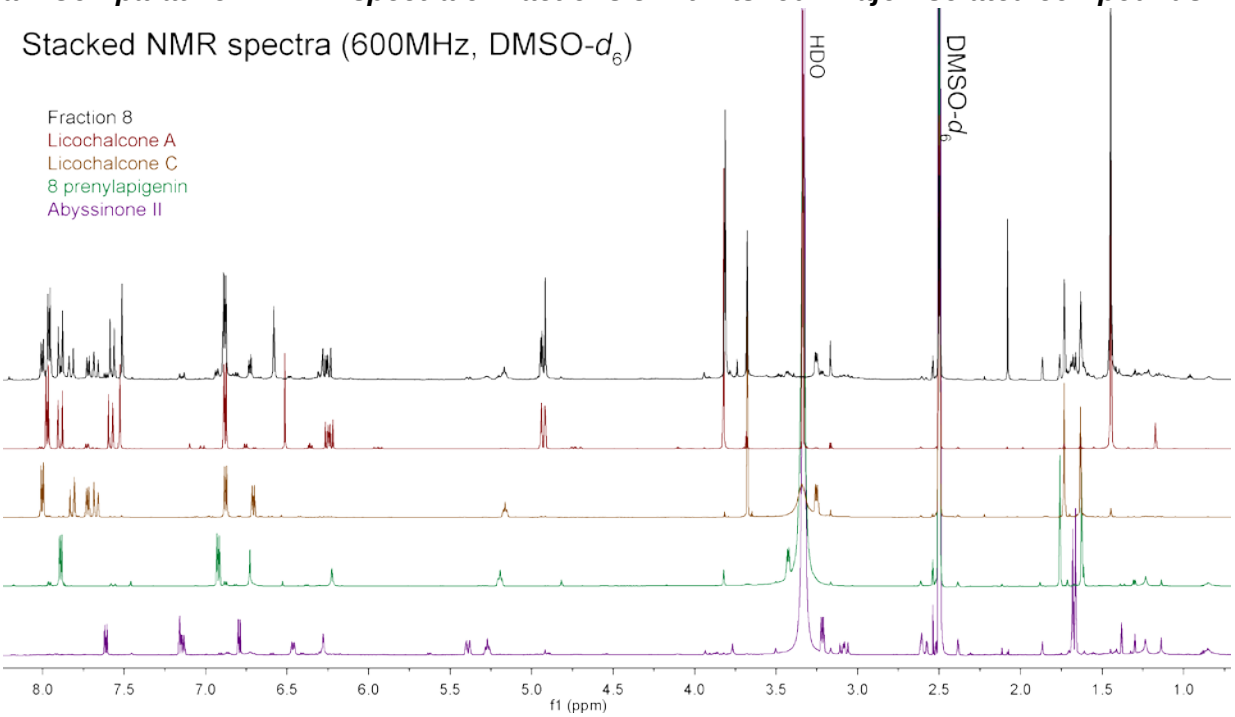
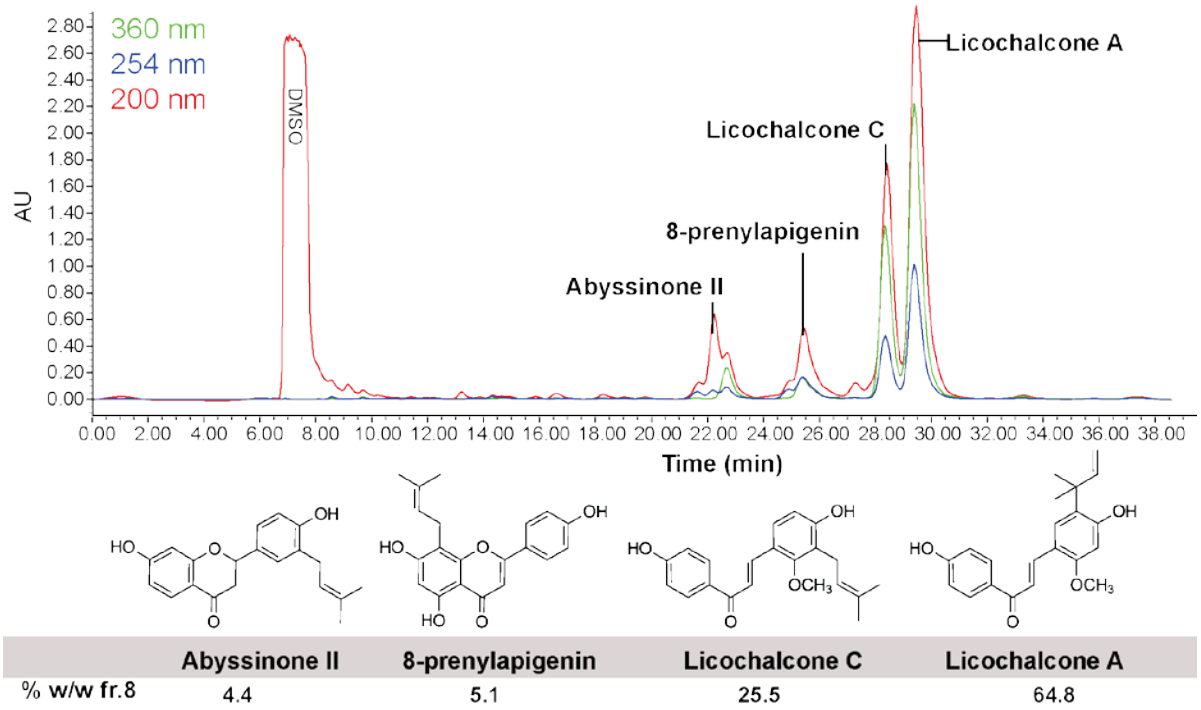
S1. ^{1}H NMR and chromatographic analyses of fraction 8 (2.1% w/w crude GI extract)

a. Comparative ^{1}H NMR spectra of fractions 8 with its four major isolated compounds



b. Semi-preparative chromatogram of fraction 8 after NMR analysis



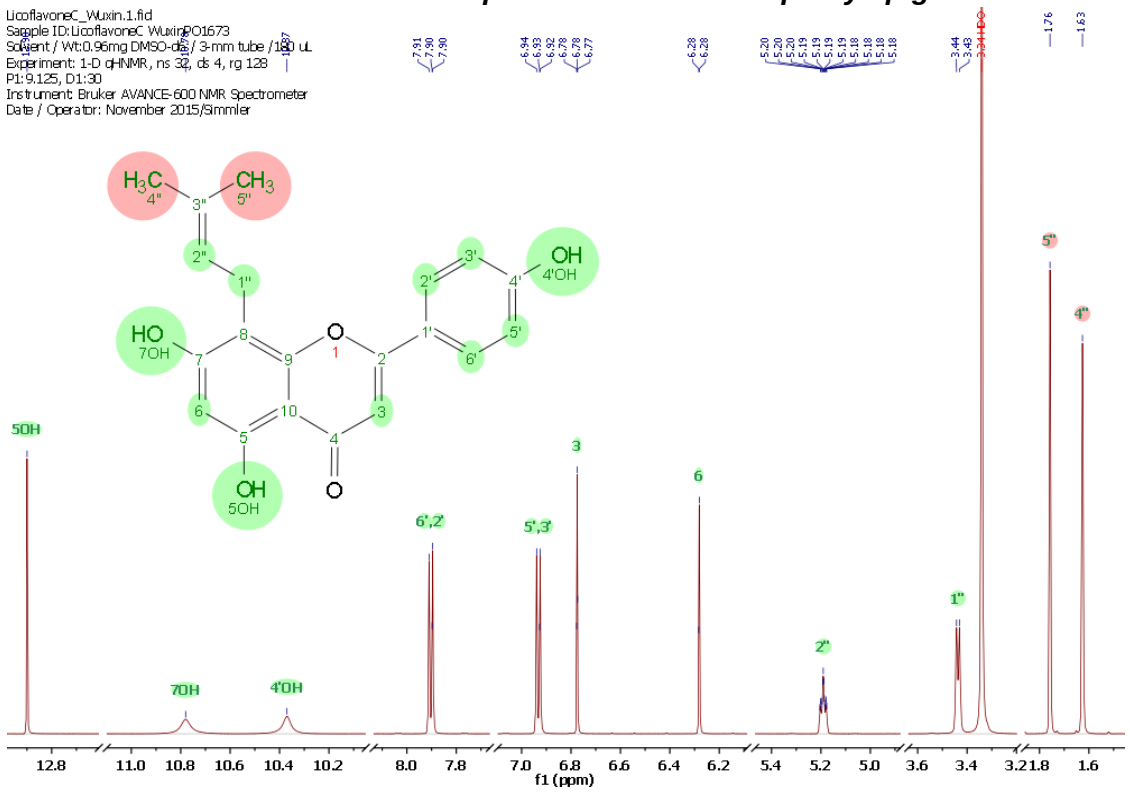
	Abyssinone II	8-prenylapigenin	Licochalcone C	Licochalcone A
% w/w fr.8	4.4	5.1	25.5	64.8

Column: YMC- Pack ODS AQ (1250 x10 mm ID, S-10 µm, 12 mm AQ12S11-251 OWT) **Gradient:** (B: Acetonitrile, A: Water) 58% B for 40 minutes in isocratic mode. Flow rate at 1.8 ml/min

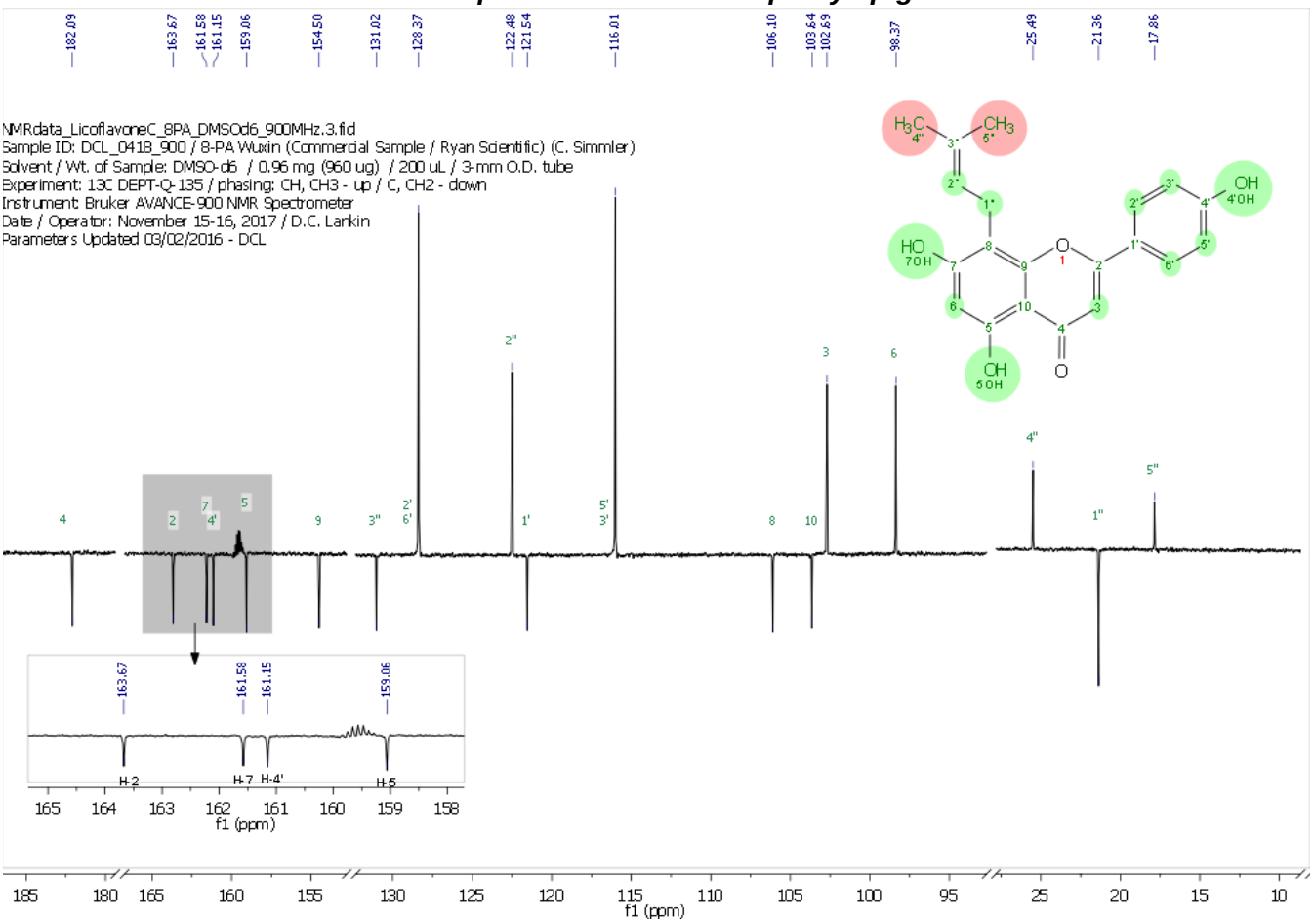
Both the semi-preparative LC-UV chromatogram and the ^{1}H NMR spectrum indicated that Licochalcone A and licochalcone C are the most abundant compounds in fraction 8, whereas 8-prenylapigenin is a minor metabolite. Considering the known fraction yield (7.13 mg = 2.1% w/w extract), the proportion of 8-prenylapigenin in fraction 8 was estimated at 5.1% w/w, and, thus, the proportion of **8-prenylapigenin in the crude extract was estimated to be ~ 0.11% w/w**.

S2. Annotated $^{1}H/^{13}C$ NMR and MS/MS spectra of 8-prenylapigenin (DMSO-d_6, 600 and 225MHz)

a. Annotated ^{1}H NMR spectrum of verified 8-prenylapigenin



b. Annotated ^{13}C NMR spectrum of verified 8-prenylapigenin

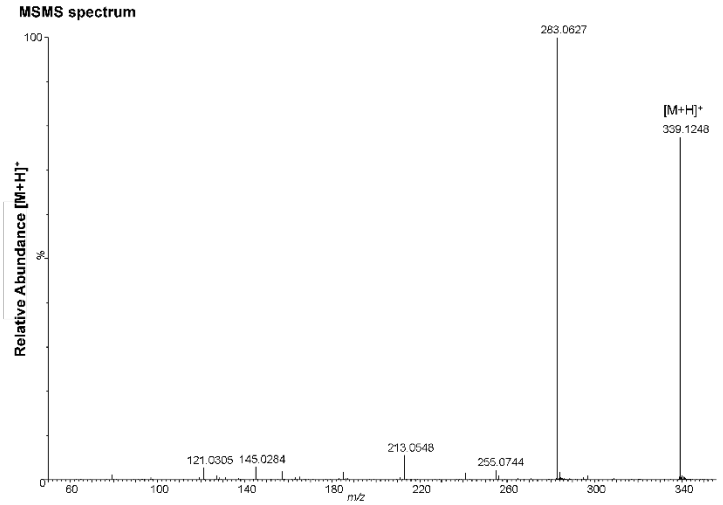


c. ^{1}H and ^{13}C NMR data of 8-prenylapigenin (DMSO-d_6, 600 MH, 225 MHz)

		8-prenylapigenin (Licoflavone C)	
Position	**mult.**	**δ_H (*J* in Hz)**	**δ_C**
C=O			181.74
2			163.73
3	1H	6.78, *s*	102.72
5 (OH)		12.85, *s*	159.10
6		6.28, *s*	98.38
7			161.64
8			106.10
9			154.49
10			103.66
1'	1H		121.54
2'/6'	2H	7.89 *AA' type* ($J_{H2'/6'-H3'/5'}$:8.63/0.24, $J_{H2'/6'-H2'/6'}$:2.88)*	127.61
3'/5'	2H	*6.92 XX' type* ($J_{H2'/6'-H3'/5'}$:8.63/0.24, $J_{H3'/5'-H3'/5'}$:2.88)*	115.17
4'	1H		161.15
1"	2H	*3.42 d* ($J_{H2"-1"}$:7.039, $J_{H1"-4"}$:0.96, $J_{H1"-5"}$:0.73)*	22.06
2"	1H	5.21 *ddqq* type ($J_{H2"-1"}$:7.04, $J_{H2"-4"}$: -1.57, $J_{H2"-5"}$: -1.37)*	122.56
3''			131.03
4" CH3 5" CH3	3H 3H	1.62 *brs* ($J_{H1"-4"}$:0.96, $J_{H2"-4"}$: -1.57)* 1.75 *brs* ($J_{H1"-5"}$:0.74, $J_{H2"-5"}$: -1.37)*	25.47 17.85
7'-<u>OH</u>		10.78 *brs*	
4'-<u>OH</u>		10.37 *brs*	

*Calculated values obtained through full spin analysis using Perch NMR software, in order to obtain the exact coupling constants.

d. MS/MS spectrum of verified 8-prenylapigenin

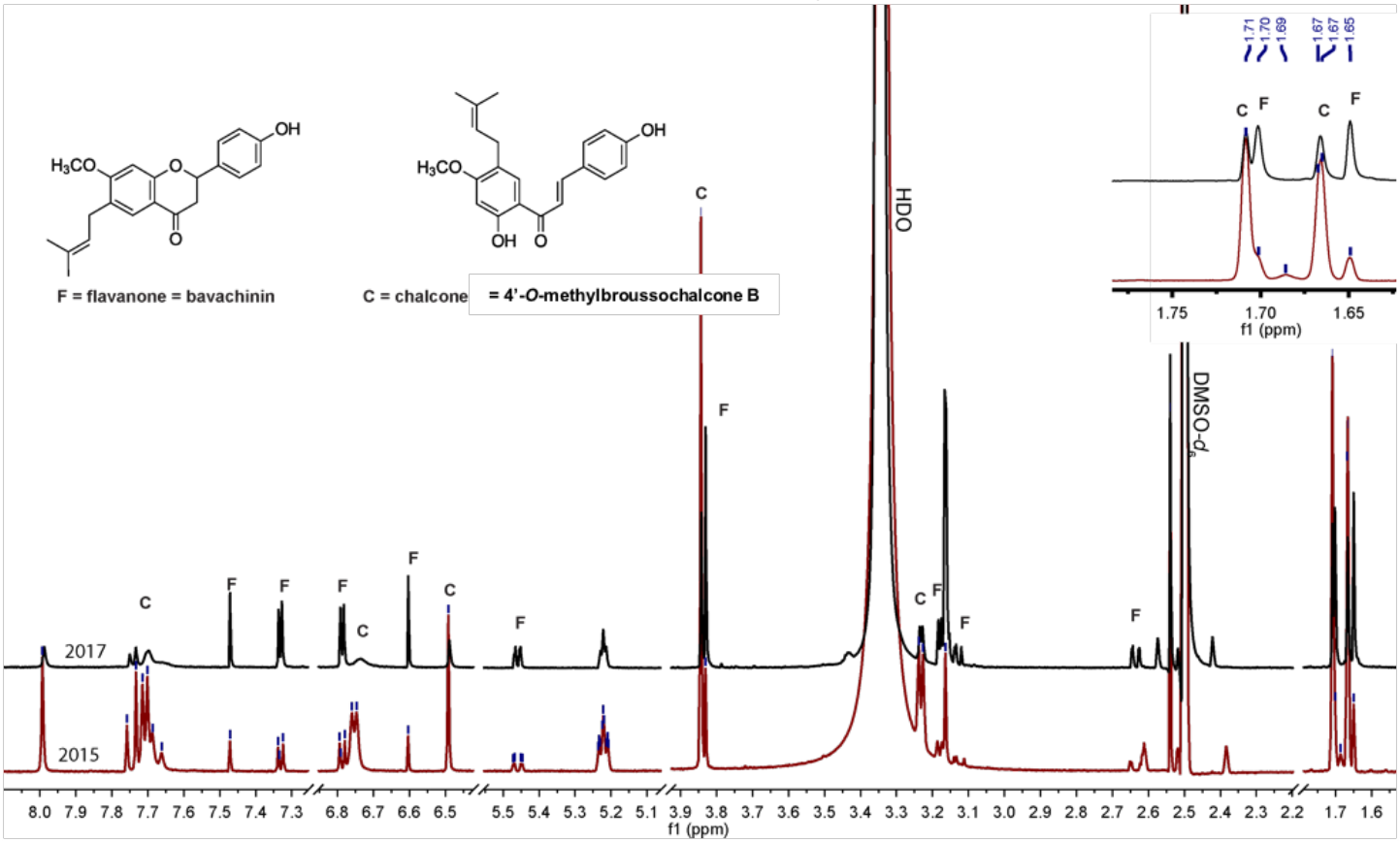


MS/MS spectrum of verified 8-prenylapigenin. The MS/MS spectrum was taken with a CE ramp between 6-50 eV in positive ionization mode.

Synonym: 8-prenylapigenin, licoflavone C
CAS Registry Number: 72357-31-4 **Molecular Formula**: $C_{20}H_{18}O_5$
Pubchem CID: 10246505 **Chemspider ID:** 8421992

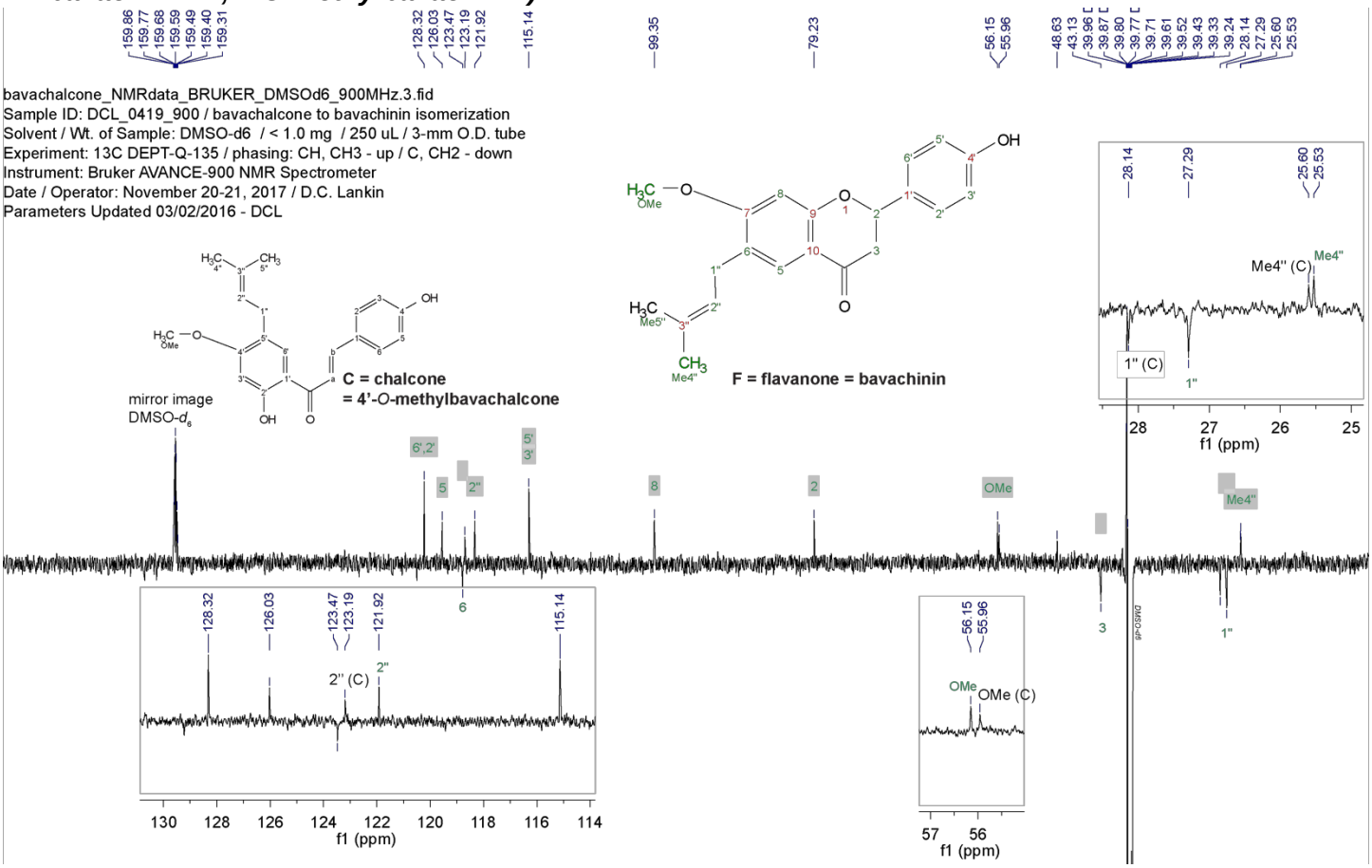
S3. Annotated ^{1}H/ ^{13}C NMR and MS/MS spectra of 4'-*O*-methylbavachalcone claimed to be 8-prenylapigenin

a. ^{1}H NMR spectra of the sample, acquired at a 2-year interval (DMSO-d_6, 600 and 900MHz)

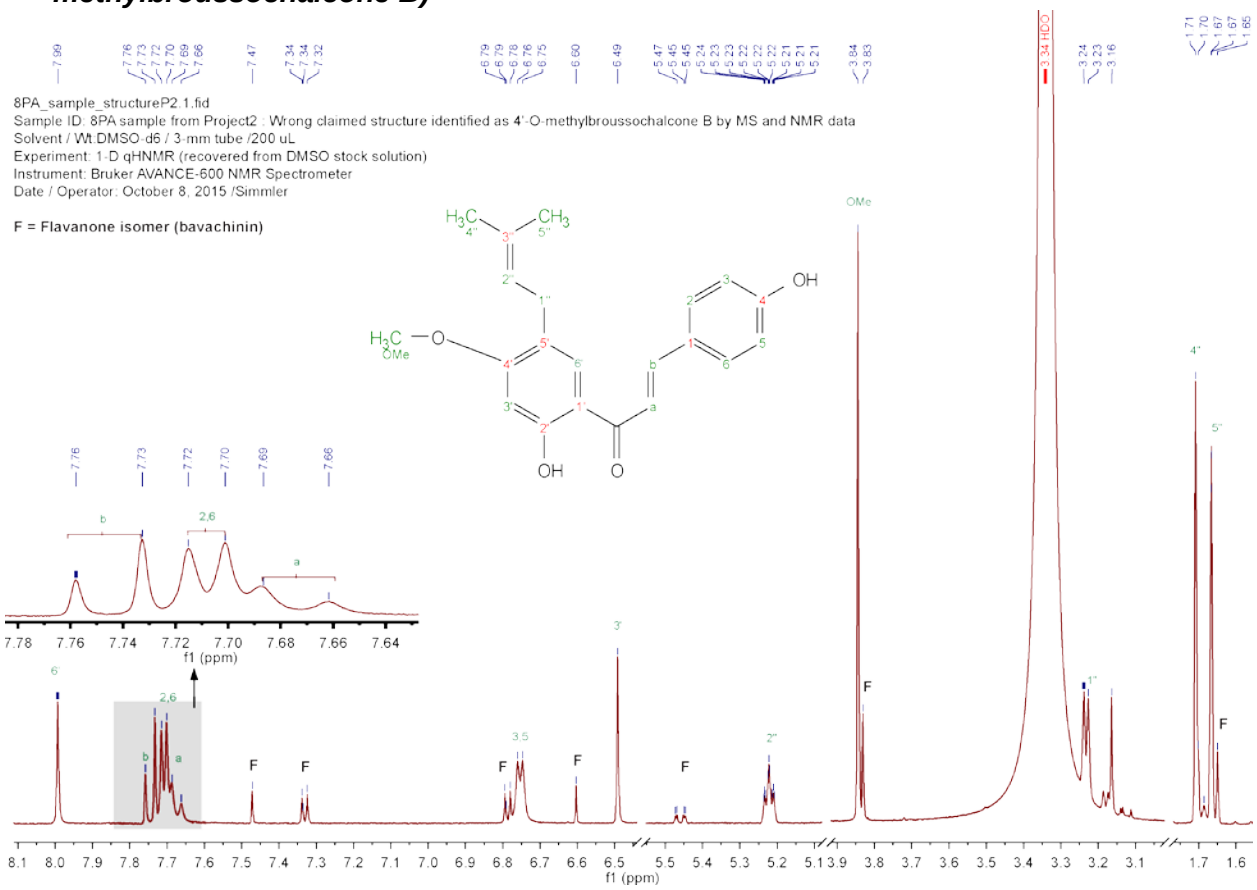


The results presented here clearly demonstrated the isomerization of the chalcone (C= 4'-*O*-methylbavachalcone or 4'-*O*-methylbroussochalcone B) into its flavanone isomer (F = bavachinin, bavachinin A or 7-*O*-methylbavachinin), occurring in the NMR tube. The ^{13}C data was acquired using the 2 year-sample and, thus, reflect only the ^{13}C resonances of the flavanone isomer

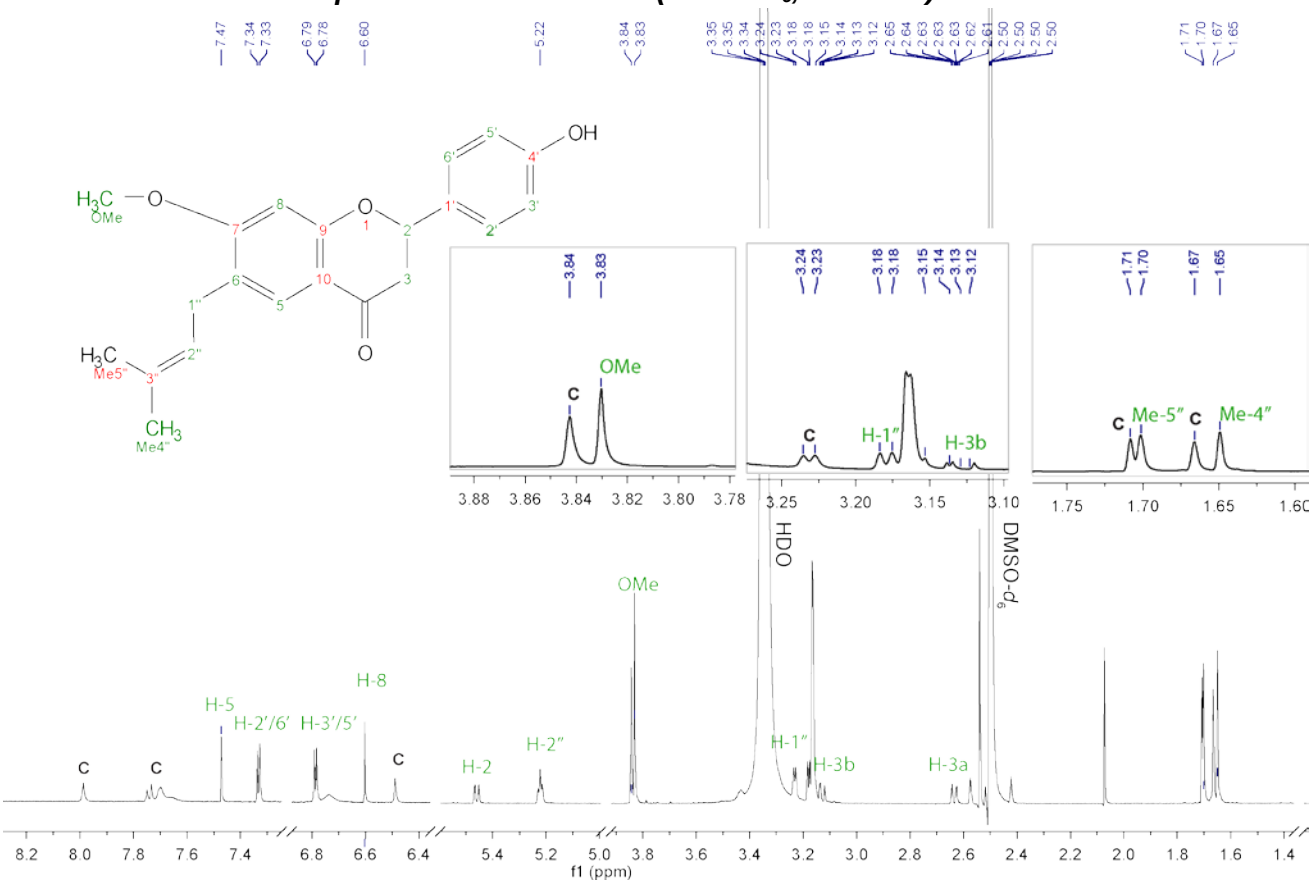
b. Annotated ^{13}C NMR spectrum (225 MHz) of the 2-year sample containing mainly bavachinin (syn. bavachinin A, 7-O-methylbavachinin)



c. Annotated ^{1}H NMR spectrum (DMSO-d_6, 600 MHz) of 4'-O-methylbavachalcone (4'-O-methylbroussochalcone B)



d. Annotated ^{1}H NMR spectrum of bavachinin (DMSO-d_6, 900 MHz)



e. ^{1}H and ^{13}C NMR data of 4'-O-methylbavachalcone and its isomer (DMSO-d_6, 600 MH, 225 MHz)

		4'-*O*-methylbavachalcone		**Bavachinin (flavanone isomer)**	
Pos.	**mult.**	**δ_H (*J* in Hz)**	**δ_C[a]**	**δ_H (*J* in Hz)**	**δ_C[b]**
C=O			191.90		nd
2		*Not present*		5.45 *dd* (J_{3a-2}:13.13; J_{3b-2}:2.84)*	79.19
3b 3a	1H 1H			3.14, *dd* (J_{3a-3b} :-16.95; J_{3b-2}:13.13)* 2.63, *dd* (J_{3a-3b} :-16.95; J_{3a-2}:2.84)*	43.13
3' (8)	1H	6.49, *s* ($J_{H3'-6'}$:0.92)	99.14	6.61, *s* (J_{8-5} :0.006)	99.14
4 (4')			nd		nd
5' (6)			121.01		123.47
6' (5)		7.99, *s* ($J_{H3'-6'}$:0.92)	130.04	7.48, *s* (J_{H5-8} :0.006)	125.95
α	1H	7.67, *d* ($J_{Hα-β}$:15.13)		*Not present*	
β	1H	7.74, *d* ($J_{Hα-β}$:15.13)		*Not present*	
1' (10)	1H		113.52		114.17
2' (9)			164.56		162.29
2/6 (2'/6')	2H	7.71 *AA'*($J_{H2/6-H3/5}$:8.29/0.80, $J_{H2/6-H2/6}$:1.69)*	131.16	7.34 *AA'* ($J_{H2'/6'-H3'/5'}$:8.15/0.62, $J_{H2'/6'-H2'/6'}$:1.71)*	128.29
3/5 (3'/5')	2H	6.75 *XX* ($J_{H2/6-H3/5}$:8.63/0.24, $J_{H3/5-H3/5}$:1.87)*	nd	*6.79 XX'*($J_{H2'/6'-H3'/5'}$:8.15/0.62, $J_{H3'/5'-H3'/5'}$:1.71)*	114.95
4' (7)	1H		163.42		164.13
O-CH3	3H	3.84, *s*	56.03	3.83, *s*	56.03
1"	2H	3.23, *d* ($J_{H2"-1"}$:7.64, $J_{H1"-4"}$:-0.80, $J_{H1"-5"}$:0.64)*	28.56	3.18, *d* ($J_{H2"-1"}$:7.45, $J_{H1"-4"}$:-0.34, $J_{H1"-5"}$:0.79)*	27.29
2"	1H	5.22, *ddqq* ($J_{H2"-1"}$:7.64, $J_{H2"-4"}$:-1.57, $J_{H2"-5"}$: -1.34)*	123.19	5.22, *ddqq* ($J_{H2"-1"}$:7.45, $J_{H2"-4"}$: -1.39, $J_{H2"-5"}$: -1.24)*	121.92
3''			131.13		132.0
4" CH3 5"CH3	3H 3H	1.67, *brs* ($J_{H1"-4"}$:-0.80, $J_{H2"-4"}$: -1.57)* 1.71, *brs* ($J_{H1"-5"}$:0.64, $J_{H2"-5"}$: -1.37)*	26.01 17.25	1.65, *brs* ($J_{H1"-4"}$: -0.34, $J_{H2"-4"}$: -1.39)* 1.70, *brs* ($J_{H1"-5"}$:0.79, $J_{H2"-5"}$: -1.23)*	26.08 17.86

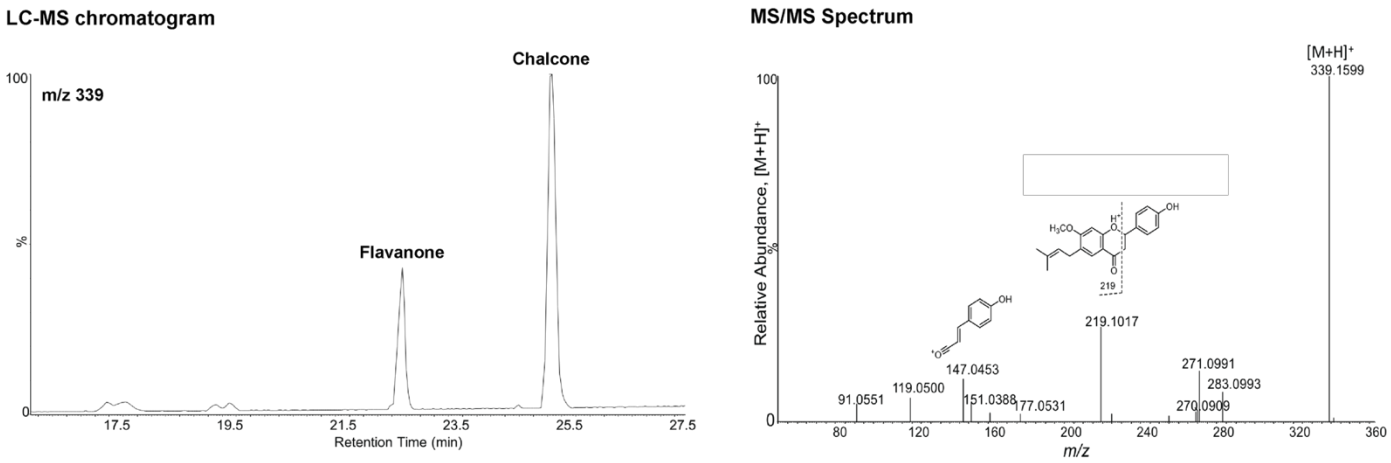
The numbers in brackets are related to bavachinin

*Calculated values obtained through full spin analysis using Perch NMR software, in order to obtain the exact coupling constants.

nd=- not detected

[a]determined from the HMBC/HSQC data, [b] and ^{13}C data for bavachinin.

f. LC-MS chromatogram of the sample received and MS/MS spectrum of 4'-O-methylbavachalcone

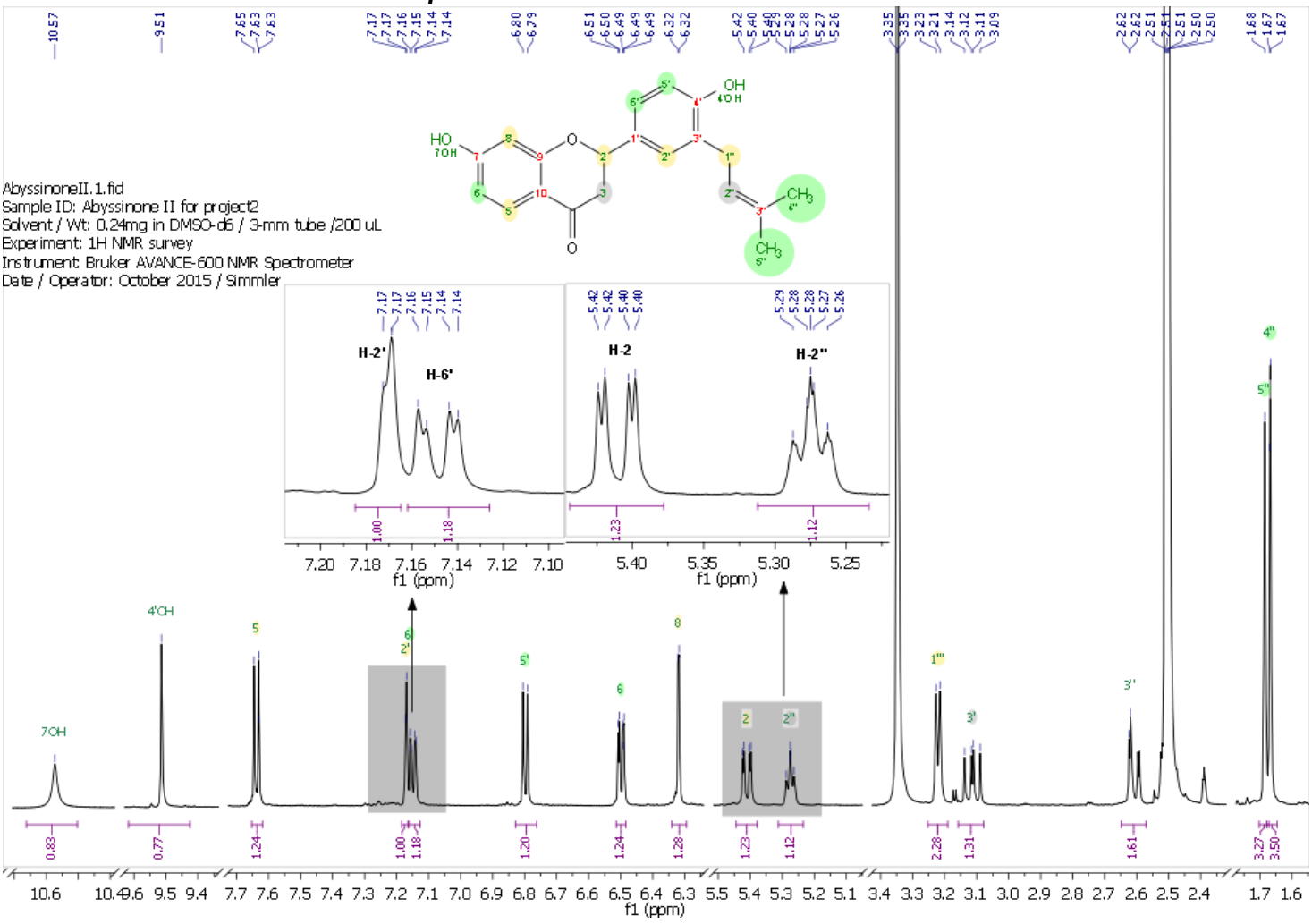


LC-MS Chromatogram (left side) and MS/MS spectrum (right side) obtained for the sample claimed to be 8-prenylapigenin. The MS/MS spectrum was taken with a CE ramp between 6-50 eV in positive ionization mode. The extracted ion chromatogram (*m/z* 339) dysplayed two peaks with the exact same MS /MS spectra, thereby suggesting the presence of two isomeric forms. F = Flavanone Bavachinin, and C = Chalcone 4'-*O*-methylbavachalcone, or 4'-*O*-methylbroussochalcone B.

Pubchem CID: 5321765 **Chemspider ID:** 4479431
CAS Registry Number: 20784-60-5 **Molecular Formula**: $C_{21}H_{22}O_4$

S4. Annotated ^{1}H NMR spectrum of Abyssinone II (DMSO-d_6, 600 MHz)

a. Annotated ^{1}H NMR spectra



b. *^{1}H and ^{13}C NMR data* (DMSO-d_6, 600-225 MHz)

Position	mult.	δ_H (*J* in Hz) in ppm	[a] δ_C in ppm
C=O			191.97
2	1H	5.41 *dd* (J_{H2-3b} :12.78, J_{H2-3a} :2.92)*	79.31
3a 3b	1H 1H	2.62 *dd* (J_{H3a-3b} :-16.76, J_{H2-3a} :2.92)* 3.11 *dd* (J_{H3a-3b} :-16.76, J_{H2-3b} :12.78)*	43.56
5	1H	7.64, *d* (J_{H6-5} : 8.22, J_{H5-8} :0.55)*	128.79
6	1H	6.50, *dd* (J_{H6-5} :8.22, J_{H8-6} 2.25)*	110.60
7 (OH)			164.80
8	1H	6.32, *d* (J_{H8-6} : 2.25, J_{H5-8} :0.55)*	102.85
9			162.29
10			114.33
1’			[b]129.24
2’	1H	7.17, *d* ($J_{H2'-6'}$: 2.4, $J_{H5'-2'}$: 0.21, $J_{H2'-1"ab}$: 0.05)*	128.75
3’			[b]127.47
4’ (OH)			156.58
5’	1H	6.80, *d* ($J_{H5'-6'}$8.66, $J_{H5'-2}$: 0.21)*	[b]114.64
6’	1H	7.15, *dd* ($J_{H5'-6'}$8.66, $J_{H2'-6'}$: 2.41)*	125.48
1”a 1”b	1H 1H	3.22 *d* ($J_{H2"-1"a}$:7.40, $J_{H1"a-4"}$:0.70, $J_{H1"a-5"}$:0.93 , $J_{H1"a-b}$:-18, $J_{H2'-1"a}$: 0.05)* 3.22 *d* ($J_{H2"-1"b}$:7.16, $J_{H1"b-4"}$:1.02, $J_{H1"b-5"}$:0.61 , $J_{H1"a-b}$:-18 $J_{H2'-1"b}$: 0.05)*	27.50
2”	1H	5.27 *ddqq* ($J_{H2"-1"a}$:7.40, $J_{H2"-1"b}$:7.16, $J_{H2"-4"}$:-1.53, $J_{H2"-5"}$: -1.28)*	123.06
3’’			132.02
4” CH3 5” CH3	3H 3H	1.68, *brs* ($J_{H1"a-4"}$:0.70, $J_{H1"b-4"}$:1.02, $J_{H2"-4"}$:-1.53) * 1.67, *brs* ($J_{H1"a-5"}$:0.93, $J_{H1"b-5"}$:0.61, $J_{H2"-5"}$:-1.28) *	26.15 18.27

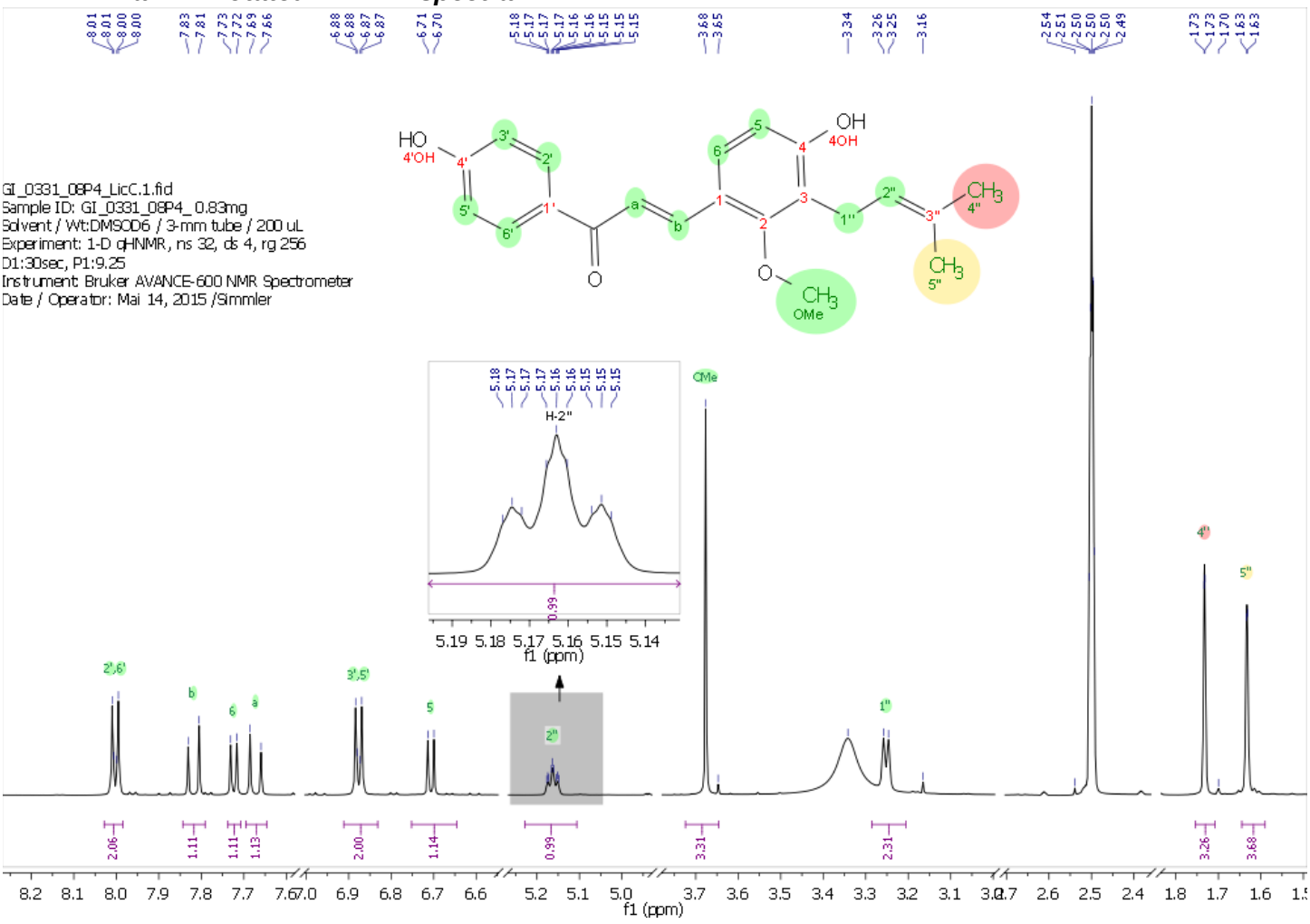
*Calculated values obtained through full spin analysis using Perch NMR software, in order to obtain the exact coupling constants.

Nd=- not detected

[a]Determined from HMBC/HSQC data, [b,13]C at 225 MHz

S5. Annotated ^{1}H NMR spectrum of licochalcone C (DMSO-d_6, 600 MHz)

a. Annotated ^{1}H NMR spectra



b. *^{1}H and ^{13}C NMR data* (DMSO-*d_6*, 600-225 MHz)

Position	mult.	δ_H in ppm (*J* in Hz)	[a]δ_C in ppm
C=O			[b]187.28
1			118.99
2 (-OMe)			158.31
3			[b]121.51
4 (-OH)			159.89
5		6.71, *d* (J_{H5-6}: 8.58)	112.32
6	1H	7.72, *d* (J_{H5-6} :8.58)	127.35
2', 6'	2H	8.00, AA' type ($J_{H2'/6'-H3'/5'}$:8.55/0.37, $J_{H2'/6'-H2'/6'}$:2.19)*	131.45
5', 3'	2H	6.88, XX' type ($J_{H2'/6'-H3'/5'}$:8.55/0.37, $J_{H3'/5'-H3'/5'}$:2.26)*	115.80
1'			129.46
4'(-OH)			162.07
α	1H	7.67, *d* ($J_{Hα-β}$ 15.56)*	119.71
β	1H	7.82, *d* ($J_{Hα-β}$ 15.56)*	138.64
1"	2H	3.25, *d* ($J_{H2"-1"}$:7.04, $J_{H1"-4"}$: 1.36, $J_{H1"-5"}$: -1.36)*	22.88
2"	1H	5.16 *ddqq* ($J_{H2"-1"}$:7.04, $J_{H2"-4"}$: 0.84, $J_{H2"-5"}$: -1.34)*	123.53
3"			130.51
-CH3 (4") -CH3 (5")	3H 3H	1.63, *brs* ($J_{H1"-4"}$: 1.37, $J_{H2"-4"}$: - 0.84) * 1.73, *brs* ($J_{H1"-5"}$: -1.36, $J_{H2"-5"}$: -1.34) *	26.11 18.06
O-CH3	3H	3.68 s	62.35

*Calculated values obtained through full spin analysis using Perch NMR software, in order to obtain the exact coupling constants.

Nd=- not detected

[a]Determined from HMBC/HSQC data, [b,13]C at 225 MHz