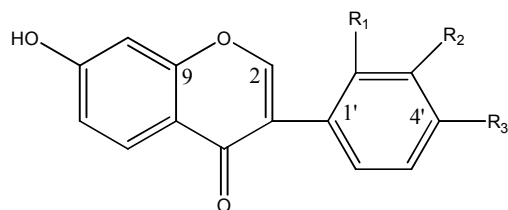


## Introduction

VG101, Bionovo's Traditional Chinese Medicine for vaginal atrophy, is an extract of three herbs. Activity-guided isolation of VG101 produced the selective estrogen receptor beta (ER $\beta$ ) compound xenogonin B (3). Upon synthesis of 3 for animal studies, it was discovered that the isolated isoflavonoid was calycosin (1), not 3. After reexamination of the NMR data and comparison of data from our isolated compound with literature data and purchased 1 and 3, we were unable to positively identify which isomer had been isolated. It was later discovered that a purchased sample of 1 was also incorrect/misidentified. Also, interpretation of the spectra was made more difficult since the chemical shifts of compounds 1-4 were drastically affected by the concentration of the analyte, the deuterated solvent used, and the addition of small amounts of benzene-d<sub>6</sub>. Other researchers have reported solvent effects in the <sup>1</sup>H NMR spectra of 2',-3',- or 4'-disubstituted, monomethoxy isoflavonoids.<sup>1</sup> In order to positively identify our isolated compound responsible for the ER $\beta$  activity, we synthesized 1-4. Our presentation will discuss the structural elucidation of compounds 1-4.

Figure 1. Four Mono-Methyl Isoflavone Analogs



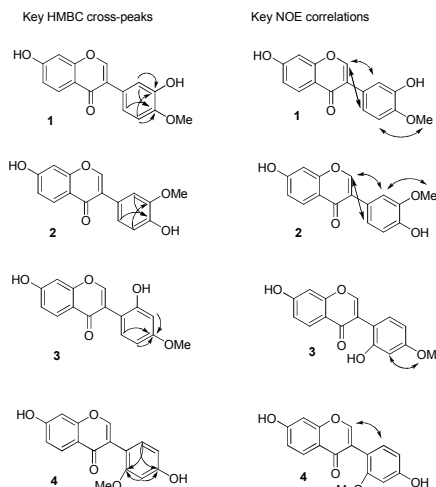
Calycosin (1)	R <sub>1</sub> =H, R <sub>2</sub> =OH, R <sub>3</sub> =OMe
Calycosin-D (2)	R <sub>1</sub> =H, R <sub>2</sub> =OMe, R <sub>3</sub> =OH
Xenogonin B (3)	R <sub>1</sub> =OH, R <sub>2</sub> =H, R <sub>3</sub> =OMe
Xenogonin B-D (4)	R <sub>1</sub> =OMe, R <sub>2</sub> =H, R <sub>3</sub> =OH

Table 1. <sup>1</sup>H and <sup>13</sup>C NMR data for 1-4

	<sup>1</sup> H				<sup>13</sup> C			
	1*	2	3	4	1	2	3	4
1								
2	8.15	8.18	7.96	8.23	152.5	152.2	153.5	155.0
3					124.3	124.2	122.1	123.1
4					174.7	174.8	174.2	177.0
5	8.03	8.04	8.00	8.05	127.5	127.5	127.3	127.4
6	6.98	6.97	6.95	7.03	115.0	115.1	114.8	115.6
7					163.0	163.0	162.8	163.0
8	6.87	6.88	6.88	6.95	102.2	102.2	102.2	102.1
9					157.9	157.8	157.9	158.4
10					117.2	117.2	117.3	116.9
1'					125.6	123.6	112.1	112.1
2'	7.19	7.27			116.5	113.2	159.3	161.3
3'			6.53	6.53	146.6	147.4	99.3	102.8
4'					147.8	147.0	158.7	157.5
5'	6.97	6.87	6.47	6.50	111.7	115.1	106.5	105.8
6'	7.03	7.06	7.08	7.22	119.9	121.7	132.3	131.9
OMe	3.85	3.87	3.71	3.78	55.5	55.5	54.9	54.7

\*NMR spectra were recorded in acetone-d<sub>6</sub> with a small amount of benzene-d<sub>6</sub>. See experimental sections for more details. Chemical shifts are in ppm.

Figure 2. Key HMBC and NOESY Cross-Peaks



## Discussion

The approximate <sup>13</sup>C chemical shifts for HO- and MeO-groups are shown in Table 2. (Predictive 13-carbon shifts in flavonoids were recently reviewed<sup>2</sup>). When two groups are *meta* to each other, as in 3-4, the substituent effects are roughly additive. However, when these groups are *ortho* to each other, the system largely breaks down because these substituents must adopt a planar orientation, with respect to the benzene ring, to be electron donating. For example, in 3-4, C-3' should be equally shielded and have ca. the same chemical shift, because, in both cases C-3' is flanked by an HO- and an MeO-group (just with positions interchanged). However, C-3' is  $\delta$  99.3 in 3 and  $\delta$  103.8 in 4. The chemical shifts of C-5' should be more different, based on substituent effects, but were actually closer ( $\delta$  105.8 and 106.5) than those of C-3' in 3-4.

NOE theory suggests one could observe NOEs between H-2 and H-2' and H-6' in compounds 1-2 and H-2 and H-6' in 3-4. In compounds 1-2, NOE cross-peaks between H-2 and H-2' and H-6' were recorded. However, in 3-4 fewer NOE cross-peaks were observed. In compound 4 an NOE between H-2 and H-6' was seen, but this NOE was not observed in 3. In addition, there was no NOE from the MeO-substituent to H-5' in 4. The NOE data in 3 contradicted the HMBC results in 3, thus, leading to the possibility of assigning the wrong structure.

Caution is necessary when using NOE data to assign substituent groups around aromatic rings in flavonoids. Using negative evidence, such as the absence of an NOE between the MeO-group and H-5' in 4, could be interpreted that the isolated compound was 3 and not 4.

Table 2: <sup>13</sup>C Chemical shift effects of different substituents<sup>a</sup>

Position	-OH	Position	-OMe
	Shift/effect (ppm) <sup>a</sup>		Shift/effect (ppm) <sup>b</sup>
<i>ipso</i>	+26.9	<i>ipso</i>	+31.4
<i>ortho</i>	-12.7	<i>ortho</i>	-14.4
<i>meta</i>	+1.4	<i>meta</i>	+1.0
<i>para</i>	-7.3	<i>para</i>	-7.7

a). From Wehrli, F.W. and T. Wirthlin. Interpretation of Carbon-13 NMR Spectra. b). to approximate the chemicals shift add/subtract this number from the  $\delta$  of benzene at  $\delta$  128.5.

**Solvents effect.** The addition of benzene-d<sub>6</sub> had a dramatic effect on the proton chemical shifts in compounds 1-4. Adding small amounts (5 drops) of benzene-d<sub>6</sub> reduced spectral crowding in calycosin (1), Figure 3. Adding larger amounts of benzene-d<sub>6</sub> (10-15 drops) led to additional resolution in some regions, but other signals were now overlapped. Other solvents were tried (THF-d<sub>5</sub>, methanol-d<sub>4</sub>, DMSO-d<sub>6</sub>), but these solvents also gave spectra with overlapping signals.

In addition, benzene-d<sub>6</sub> affected the proton shifts at H-2 located on the C ring. In compounds 1-4, when pure acetone-d<sub>6</sub> was used, the <sup>1</sup>H chemical shift of H-2 was located downfield of the large coupling doublet, H-5. Three titrations (5, 10, 15, drops) with benzene-d<sub>6</sub> were performed, and a <sup>1</sup>H spectra was recorded. The first titration showed a small downfield shift in H-2. After the second titration, H-2 and H-5 were overlapped, and after the third titration, H-2 was shifted downfield of H-5 by a 0.02-0.05 ppm. This effect is shown in Figure 4.

Figure 3. (A) NMR of 1 without benzene-d<sub>6</sub>, (B) NMR of 1 with benzene-d<sub>6</sub>.

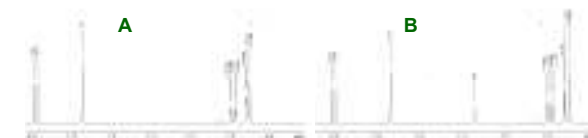
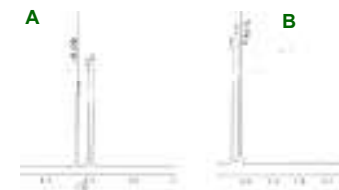


Figure 4. (A) NMR of 1 with benzene-d<sub>6</sub>, (B) NMR of 1 without benzene-d<sub>6</sub>.



## Experimental and Isolation

**Experimental.** NMR spectra were acquired on a Varian 400 MHz instrument using methanol-d<sub>4</sub>, acetone-d<sub>6</sub> and benzene-d<sub>6</sub>. The NMR data in Table 1, as well as the 2D data shown in Figure 2, were acquired by mixing 4 mL of acetone-d<sub>6</sub> with 1 mL of benzene-d<sub>6</sub>. Ten milligrams of 1-4 were dissolved in 600  $\mu$ L of the previously described deuterated solvent mixture. <sup>1</sup>H, <sup>13</sup>C, DEPT, COSY, HSQC, HMBC, and NOESY spectra were acquired using standard pulse sequences supplied by Varian. The NOESY mixing time was 0.5 and 1.0 seconds. In addition, 1D NOE spectra were acquired with mixing times of 0.5, 0.7, 0.9 and 1.0 seconds. Preparative HPLC used a Waters 2545 auto purification system with a 2487 UV detector at 254 nm. Preparative HPLC was carried out on a Waters Sunfire 19.0 x 150 mm, 5  $\mu$ m column. Column chromatography used Merck silica gel, 230-400  $\mu$ m, and TLC was performed on Merck 250  $\mu$ m thick plates.

**Isolation.** The herbs were exhaustively extracted with 8:2 ethanol(95%)-water and concentrated *in vacuo*. The extract was sequentially partitioned with hexane and ethyl acetate (EtOAc). The ethyl acetate layer was chromatographed over silica gel using mixtures of hexane and EtOAc (0.1 to 1.0, hexane—EtOAc in 10% steps). Next, the fraction containing the active compound was subject to reversed-phase preparative HPLC, 10% B to 100% B in 20 min, flow 20 mL/min, where solvent A was 10 mM ammonium acetate and solvent B was MeCN. Preparative HPLC gave a fraction that was approximately 60% 1. Final purification of 1 was achieved by silica gel TLC developed with 4:6 benzene—EtOAc with 0.5 mL of formic acid added per 50 mL.

**Synthesis.** Isoflavonoids 1-4 were synthesized using established methods. Briefly, the appropriate MOM protected aldehyde was condensed using KOH in MeOH/H<sub>2</sub>O. Next, each chalcone was rearranged using Ti(NO<sub>3</sub>)<sub>3</sub> in MeOH followed by cyclization and deprotection of MOM with HCl in MeOH.

## References

- Du, Xingang, *et al.*, Solvent effect in <sup>1</sup>H NMR spectra of 3'-hydroxy-4'-methoxy isoflavonoids from *Astragalus membranaceus* var. *mongholicus*. *Magn. Reson. Chem.* 2006; **44**: 708-712.
- Burns, D., *et al.*, A predictive tool for assessing <sup>13</sup>C NMR chemical shifts of flavonoids. *Magn. Reson. Chem.* 2007; **45**: 835-845.