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Identification of New Urinary Metabolites of Byakangelicin, a Component of *Angelicae dahuricae Radix*, in Rats

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Byakangelicin, 9-(2,3-dihydroxy-2-methylbutoxy)-4-methoxy-7*H*-furo[3,2-g][l]benzopyran-7-one (BKG), a component of *Angelicae dahuricae Radix*, is considered to be an inhibitor of aldose reductase for the treatment of diabetic cataract. An analytical method for the isolation of BKG developed by high-performance liquid chromatography has been reported. No literature on the metabolism of BKG, however, has been found. With the purpose of identifying new metabolites of BKG, BKG (100 mg/kg) was orally administered to Sprague-Dawley rats via a gavage. Using a metabolic cage, urine was collected for 24 h, and the urine samples were extracted by liquid-liquid extraction. For structural identification of new urinary metabolites of BKG, various instrumental analyses were conducted by gas-chromatography/mass spectrometry, high-performance liquid chromatography/diode array detector, liquid chromatography/mass spectroscopy with thermospray interface and ^{1}H nuclear magnetic resonance spectroscopy. Two metabolites produced from the *O*-demethylation or *O*-dealkylation of BKG were newly identified, and another new but unknown metabolite was assumed to be the hydroxylated form of BKG. These results indicate that the major metabolic products of BKG are formed by O-demethylation or *O*-dealkylation of BKG side chains.

Key words: Byakangelicin, *Angelicae dahuricae,* Urinary metabolites, Metabolism, Rat, *O*-Demethylation, *O*-Dealkylation

INTRODUCTION

Byakangelicin (9-(2,3-dihydroxy-2-methylbutoxy)-4-methoxy-7*H*-furo[3,2-g][l]benzopyran-7-one; BKG), a naturally occurring constituent of *Angelicae dahuricae Radix* has been investigated and reported (Oh *et al.*, 2002; Kim *et al.*, 2002; Shin *et al.*, 1990) to be an inhibitor of aldose reductase for the treatment of diabetic cataract (Shin *et al.*, 1994). BKG is considered to be a naturally potent inhibitor for aldose reductase and may be applied to the development of treatment for diabetic cataract. Diabetes results in a variety of tissue and functional changes, i.e., neuropathy, retinopathy, nephropathy, and cataract formation. Although the pathogenesis of these long-term diabetic complications remains unknown, mounting evidence indicates that their onset and severity are linked to the intracellular accumulation of sorbitol, which results from glucose catalyzed by aldose reductase (Tomlinson *et al.*, 1994; Terashima *et al.*, 1984). Although various synthetic inhibitors of aldose reductase have been reported to prevent cataract by reducing aldose reductase activity, these inhibitors exerted severe toxicity and side effects (Kador, 1988). Various structurally diverse inhibitors of aldose reductase such as chromone, benzoxazine, flavone, benzo[d,e]isoquinoline, coumarin, xanthone, 11-oxo-11*H*-pyrido[2,1-b]quinazoline, naphthalene, 3-oxo-3*H*-phthalazine, zopolrestat, rhodamine, FR74366 and sorbinil have been developed (Beyer-Mears *et al.*, 1996; Ao *et al.*, 1991; Beyer-Mears and Cruz, 1985; Saiki *et al.*, 1971).

Methanol extract of *Angelicae dahuricae* resulted in the isolation of six coumarins including (+)-BKG that exhibited strong hepatoprotective activity (Oh *et al.*, 2002). Furanocoumarin analogues such as BKG and isopimpinellin also have inhibitory effects either on insulin-stimulated lipogenesis (Kimura *et al.*, 1982) or hepatic microsomal drug metabolism enzymes (Shin and Woo, 1986). Recently, the X-ray crystal

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structure of BKG was determined (Kim *et al.*, 2002). An analytical method for the isolation of BKG from *Angelicae dahuricae Radix* developed by high-performance liquid chromatography has been reported (Shin *et al.*, 1990). No literature on the metabolism of BKG is available yet. In this experiment, a metabolism study of BKG was conducted using various instrumental analysis methods in the urine of rats orally administered BKG.

MATERIALS AND METHODS

Chemicals

BKG (from *Angelicae dahuricae Radix,* as shown in Fig. 4) and isoimperatorin were supplied by Dr. K. H. Shin of the Natural Products Research Institute, Seoul National University. β-Glucuronidase/arylsulfatase were purchased from Boehringer Co. (Peterburg, VA, USA). Methanol, acetone, and diethyl ether were obtained from J. T. Baker (Phillipsburg, NJ, USA). All other chemicals were of analytical grade.

Animals

Adult Sprague-Dawley male rats (2-months-old, 200± 50 g) were purchased from Dae-Han Laboratory Animal Research Center (Eumsung, Chungbuk, Korea). The rats were allowed free access to food (Samyang Co., Seoul, Korea) and tap water, and maintained on a 12/12 h light/dark cycle.

Byakangelicin administration to rats

BKG-free blank urine was collected one day before administration to rats using a metabolic cage (Nalgene, NY, USA). BKG (100 mg/kg) dissolved in aqueous solution of 1% carboxymethyl cellulose was orally administered to rats via a gavage. Urine was collected for 24 h.

Gas chromatography-mass spectrometry/electron impact (GC-MS)

A gas chromatography-mass spectrometer (5890A/5970B, Hewlett-Packard, CA, USA) equipped with a methylsilicone capillary column (HP-1, 17 m length × 0.2 mm inner diameter × 0.3 μm film thickness) was used. The flow rate of helium as the carrier gas was 0.7 mL/min. The split ratio was 10: 1 and the flow rate of the septum purge vent was 4 mL/ min. The injector of the gas chromatograph and transfer line of the ion source were set to 280°C and 300°C, respectively. The initial oven temperature of 160°C, was increased by 20°C per minute to 300°C, and then held for 7 min. The ionization potential of the electron impact mode of the mass selective detector was 70 eV.

High-performance liquid chromatography with a diode array detector (HPLC/DAD)

A Hewlett-Packard HPLC (HP 1090M Series) with a diode array detector equipped with μ-Bondapak C_{18} (10 μm particle size, 125 Å pore size, 3.9×150 mm; Waters, Milford, MA. USA) was used. The selected DAD wavelength was ranged from 200 to 400 nm. The column oven temperature was set at 40°C. The mobile phase consisted of water/acetonitrile (10:90, v/v%) and was isocratically operated. HPLC fractions of metabolites **M1** and **M2** were repeatedly collected and freeze-dried overnight to obtain the NMR spectra.

Liquid chromatography-mass spectrometry (LC/MS)

LC/MS (Hewlett-Packard 1090 LC/HP5890 MS) combined with HP thermospray interface was used. The system was connected to a ChemStation (HP 59940, HP-UX series) and disc drive (HP7946).

^{1}H-Nuclear magnetic resonance spectroscopy (NMR)

^{1}H-NMR spectra ($CDCl_3$) were recorded on a Varian Gemini 300 spectrometer. The chemical shift was presented in ppm.

Extraction of byakangelicin and its metabolites in free- and conjugated-fractions of urine

Urine (3 mL) was added to a centrifuge tube and the urine pH value was adjusted to 2.0 with 1 N HCl. After addition of isoimperatorin as the internal standard and 5 ml of diethyl ether, the tube was vigorously mixed on a shaker (Buchler, Germany). The tube was centrifuged at 600 g for 10 min and the organic layer was transferred to tubes by freezing the tube in a refrigerating circulator (-25 to -30°C, 3-5 min; Lauda, Germany). This step was repeated and the organic layer was combined. The organic layer was evaporated on an evaporator *in vacuo.* Subsequently, to the aqueous layer, 100 μL of β-glucuronidase/arylsulfatase (5.5 and 2.5 units/mL, respectively) was added. The pH value of the reaction mixture was 5.2. The tube was hydrolyzed by incubating at 37°C for 12 h in a heating block (Dri-block, Techne Inc., Princeton, NJ, USA), extracted twice with 5 mL of diethyl ether by a shaker and centrifuged at 600 g for 10 min. The organic layer was evaporated. The residue obtained from free- or conjugated-fractions was dissolved in 100 μL of methanol, and the solution was injected into the GC/MS, HPLC/DAD and LC/MS systems for identification of BKG metabolites. The HPLC/ DAD fractions collected for the metabolites **M1** and **M2** were freeze-dried and applied to ^{1}H-NMR.

RESULTS AND DISCUSSION

Gas chromatography-mass spectrometry (GC/MS)

Total ion chromatograms of GC/MS obtained from the free (Fig. 1, A) and conjugated (Fig. 1, B) fractions of the

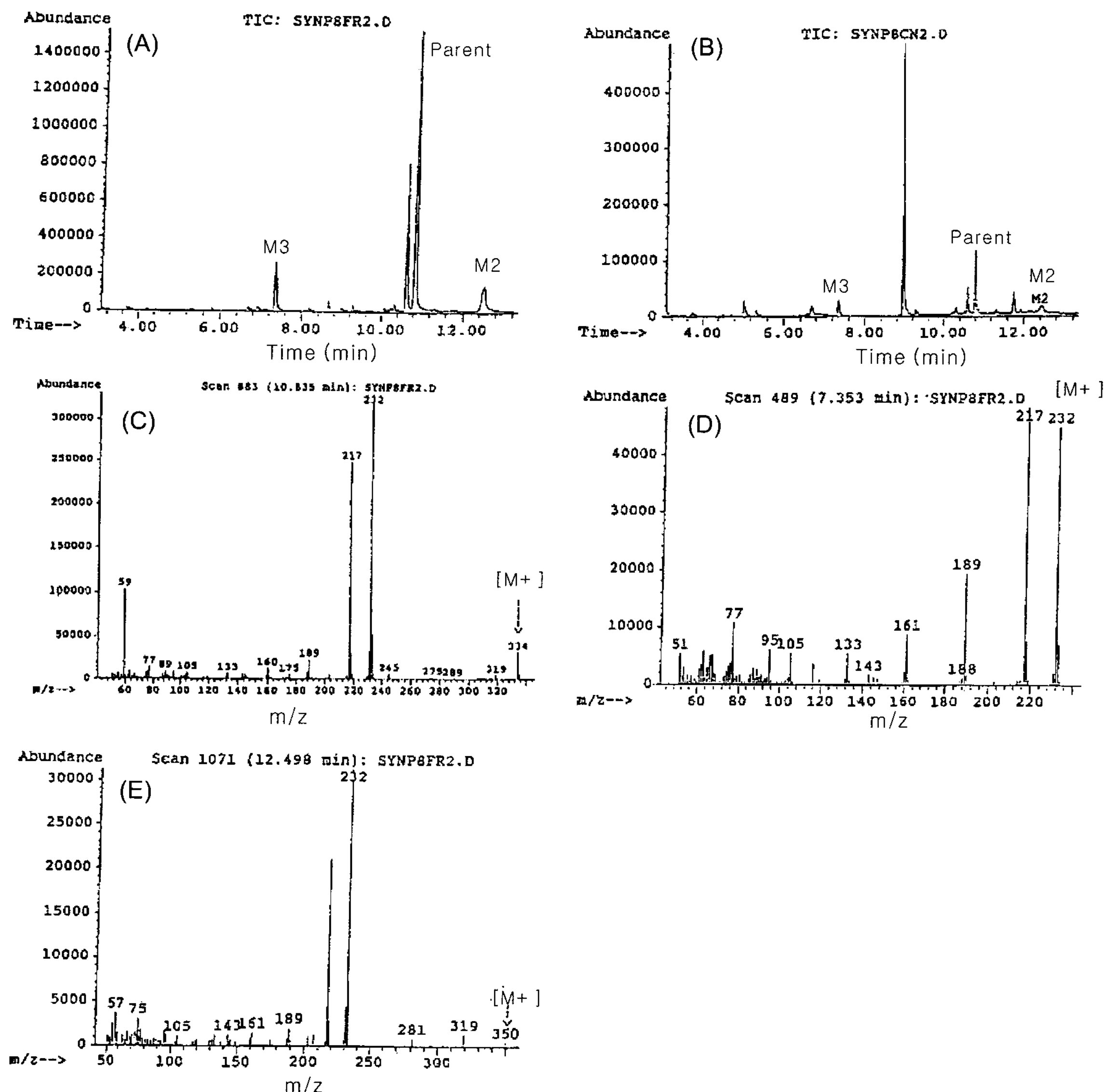

Fig. 1. Total ion chromatograms and mass spectra for byakangelicin and its metabolites obtained by GC/MS from rat urine samples after oral administration of byakangelicin (100 mg/kg). The total ion chromatograms were obtained from the free (A) and conjugated fractions (B) of the rat urine. The parent byakangelicin (10.9 min) and its urinary metabolites of **M3** (12.5 min) and **M2** (7.3 min) were detected in either free- or conjugated-fractions extracted with diethyl ether from the rat urine. The GC/MS spectra of byakangelicin (C), and the metabolites **M3** (D), and **M2** (E) are presented.

rat urine sample after oral administration of 100 mg/kg BKG are shown in Fig. 1. The same metabolites were found in the free- and conjugated-fractions. The retention times of the parent BKG and metabolites **M2** and **M3** were 10.9, 12.5 and 7.3 min, respectively. The parent BKG and two unknown metabolites (**M2** and **M3**) showed qualitatively higher abundance in the free fraction than in the conjugated fraction. The identical metabolites were also observed in the mice urine (data not shown). From the mass spectra of the parent compound BKG, as shown in Fig. 1C, BKG showed the characteristic mass ions of m/z 232 (base peak), m/z 334 (M^+, molecular weight), and m/z 59 (β-cleavage in its 9-(2,3-dihydroxy-2-methylbutoxy) moiety). In the mass scan spectra of **M3** and **M2** (Figs. 1D and E, respectively), the common characteristic ions for the two metabolites were m/z 217 and m/z 232. The molecular weight of metabolite **M2** was observed to be m/z 350, which was coincident with the data obtained from LC/MS.

High-performance liquid chromatography/mass spectrometry

The HPLC chromatograms and LC/MS spectra for the parent BKG and its two metabolites (**M1** and **M2**) are

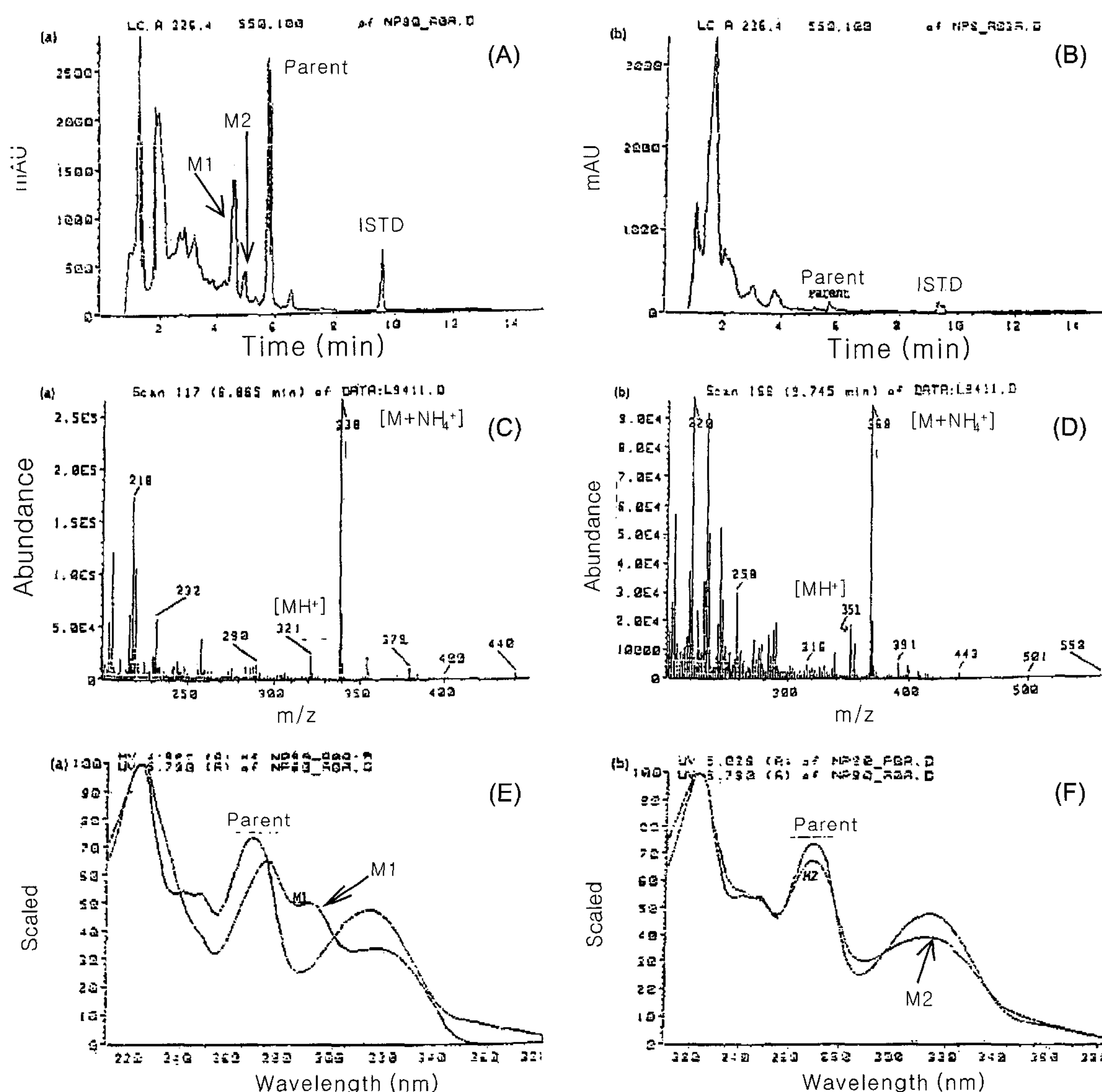

Fig. 2. The HPLC chromatograms, LC/MS spectra, and HPLC/DAD scan spectra of byakangelicin and its urinary metabolites. The HPLC chromatogram was obtained with a diode array detector of free fraction from the rat urine after oral administration of 100 mg/kg byakangelicin (A). The HPLC chromatogram (B) was obtained from byakangelicin-free urine blank which spiked the authentic standard byakangelicin (10 μg). The LC/MS spectra of metabolites **M1** and **M2** are shown in the panels C and D. The characteristic ions of [MH$^+$] and [M+NH$_4^+$] are indicated at the corresponding peaks. The HPLC/DAD spectra of byakangelicin (parent) and its metabolites (**M1** and **M2**) detected with a diode array detector are shown in E and F, respectively, where the HPLC/DAD spectra of metabolites are compared to that of the parent byakangelicin.

shown in Fig. 2. The retention time of the parent BKG was 5.7 min and that of the metabolites was 4.6 min for **M1** and 4.9 min for **M2**. Based on these retention times obtained by reverse-phase HPLC, the polarity of the compounds was in the order of **M1** (number of OH group: 3) > **M2** (number of OH group: 3) > BKG (number of OH group: 2). From the LC/MS spectra of **M1** and **M2** separated from the HPLC, the molecular ion of **M1** was observed to be m/z 320 (m/z 321 [MH$^+$]; m/z 338 [M+NH$_4^+$]), and the molecular ion of **M2** to be m/z 350 (m/z 351 [MH$^+$]; m/z 368 [M+NH$_4^+$]), as shown in Fig. 2C and D), respectively. The metabolite **M3** detected from GC/MS was not found in HPLC.

HPLC/Diode array detector

The HPLC/DAD absorption spectra detected by a photodiode array detector are presented in Fig. 2 (E and F), where the UV spectra of the parent BKG are compared to those of the metabolites. While the maximum wavelength of the metabolite **M1** around 270 nm was shifted to the right compared to that of the parent compound, the UV spectrum of the metabolite **M2** was very similar to that of the parent BKG. This data indicates that the shift of wavelength shown in the UV spectra of **M1** may be due to

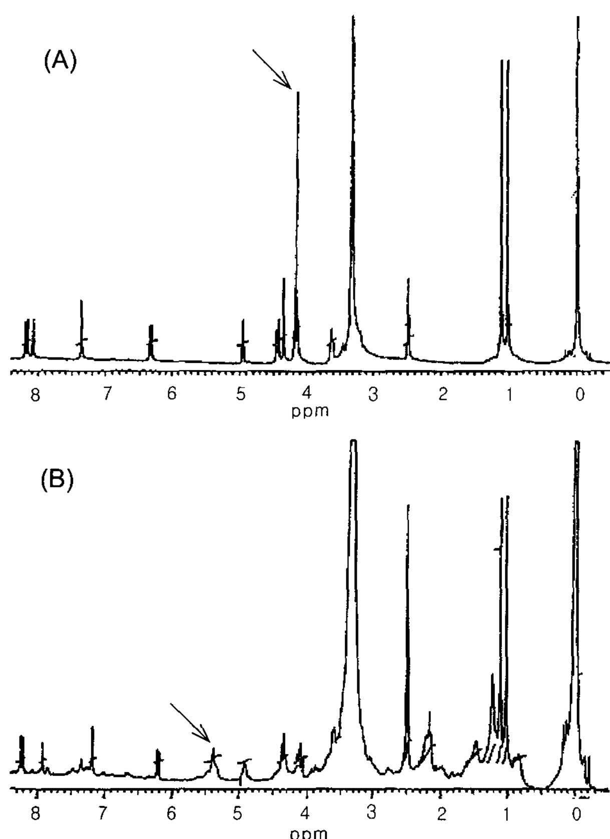

Fig. 3. ^{1}H-NMR spectra of byakangelicin (A) and its metabolite **M1** (B). The methoxy group (which appeared at δ=4.16 ppm) in the spectra of the metabolite **M1** (B) was not present at δ=4.16 ppm, compared to that of the parent compound shown in the panel A. The hydroxy group (δ=5.42 ppm) was present in the ^{1}H-NMR spectra of the metabolite **M1** (B).

structural changes of the chromophore of **M1**, rather than that of the side chain of the BKG structure.

^{1}H-Nuclear magnetic resonance spectroscopy (NMR)

The metabolite fractions obtained from HPLC/DAD were collected and applied to NMR. The NMR spectra for BKG and its metabolite **M1** are presented in Fig. 3. The major difference between the parent BKG and the metabolite **M1** occurred in the 4-methoxy and 9-(2,3-dihydroxy-2-methylbutoxy) moieties of the BKG chemical structure, where **M1** does not possess the 4-methoxy group (δ=4.16 ppm) but has a hydroxy group (δ=5.42 ppm), compared to the chemical structure of the parent compound. The concentrations of metabolite **M2** from its HPLC fractions were not high enough to obtain NMR spectra. These results are schematically summarized in Fig. 4 and Table I.

Taken together these results demonstrated that the metabolite **M1** of BKG was produced by *O*-demethylation at C-4 (molecular weight, 320), the metabolite **M3** resulted from *O*-dealkylation at C-9 (molecular weight, 232), and

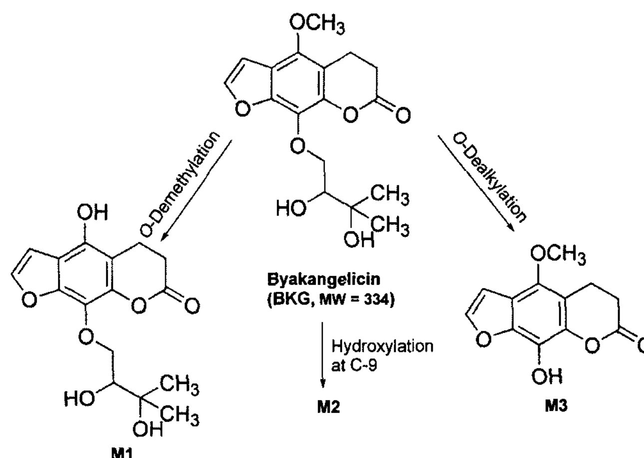

Fig. 4. The metabolic pathways of byakangelicin and summary of analytical data. **M1**, O-demethylation of byakangelicin; **M2**, possible hydroxylated metabolite in a side chain of byakangelicin; **M3**, *O*-dealkylation of byakangelicin.

Table I. Summary of the analytical data from GC/MS, LC/MS, HPLC /DAD and NMR

	M1	M2	M3
Detection	LC/MS, NMR HPLC/DAD	LC/MS, GC/MS HPLC/DAD	GC/MS –
Molecular weight			
(Calculated)	320	350	232
(Observed)			
GC/MS: [M$^+$]	–	350	232
LC/MS: [M+H$^+$]	321	351	–
[M+NH$_4^+$]	338	368	–
NMR	5.42 ppm	ND	–
No. of OH groups	3	3	1

No., number; ND, Not detected.

the metabolite **M2** may be produced by hydroxylation of BKG at C-9 (molecular weight, 350).

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