

Metabolism of Diosbulbin B In Vitro and In Vivo in Rats: Formation of Reactive Metabolites and Human Enzymes Involved[§]

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Received March 25, 2014; accepted July 21, 2014

ABSTRACT

Diosbulbin B (DB), a major constituent of the furano-norditerpenes in *Dioscorea bulbifera* Linn, exhibits potential antineoplastic activity and hepatotoxicity. The metabolism and reactive metabolites of DB in vitro (with human and animal liver microsomes) and in vivo in rats were investigated. The human enzymes involved in DB metabolism were identified. DB was first catalyzed into reactive metabolites of 2-butene-1,4-dial derivatives dependent on NADPH and then trapped by Tris base or oxidized to hemiacetal lactones (M12 and M13) in microsomal incubations. Tris base was used as buffer constituent and as a trapping agent for aldehyde. Methoxylamine and glutathione (GSH) were also used as trapping agents. DB metabolism in vivo in rats after oral administration was consistent with that

in vitro. The structures of M12 and M13, as well as mono-GSH conjugates of DB (M31), were confirmed by nuclear magnetic resonance spectroscopy of the chemically synthesized products. The bioactivation enzymes of DB were identified as CYP3A4/5, 2C9, and 2C19. CYP3A4 was found to be the primary enzyme using human recombinant cytochrome P450 enzymes, specific inhibitory studies, and a relative activity factor approach for pooled human liver microsomes. Michaelis-Menten constants K_m and V_{max} were determined by the formation of M31. The reactive metabolites may be related to the hepatotoxicity of DB. The gender difference in CYP3A expression in mice and rats contributed to the gender-related liver injury and pharmacokinetics in mice and rats, respectively.

Introduction

Diosbulbin B (DB) is a major constituent of the furano-norditerpenes in *Dioscorea bulbifera* Linn, which has been used in traditional Chinese medicine as a remedy for sore throat, struma, and tumor (Zhang et al., 2008). Severe liver injuries, including liver failures, and even death have been reported in patients who took *D. bulbifera* for more than eight consecutive days (Niu and Chen, 1994). Many diosbulbins, which include diosbulbin A–H (Kawasaki et al., 1968; Ida et al., 1978), diosbulbin I–J (Wang et al., 2009), and diosbulbin K–M, have been separated and identified from *D. bulbifera* (Liu et al., 2010). DB has been confirmed as one of the main hepatotoxic constituents in *D. bulbifera*. This compound also causes gender-related liver injury in mice (Wang et al., 2011) as well as gender-related pharmacokinetic behavior in rats (Yang et al., 2013). DB also exhibits remarkable antitumor activity in S180 cell-bearing mice (Komori, 1997; Wang et al., 2012), but no activity in a tumor cell line in vitro (Komori, 1997). These data imply that DB metabolism plays an important role in the pharmacological activity and hepatotoxicity of DB in vivo.

Xenobiotics are usually converted to chemicals that can be more readily eliminated from the body by metabolic biotransformation once they enter the body. Xenobiotic metabolites may be classified into the following broad classes according to their pharmacological properties: 1) inactive metabolites, which possess no pharmacological activity; 2) active metabolites, which may have pharmacological properties of lower, equal, or greater magnitude than their parent compounds; and 3) reactive metabolites that can covalently react with and alter the functional macromolecular structure of endogenous targets in vivo, resulting in unwanted toxic effects (Njuguna et al., 2012). For example, teucrin A, a neo-clerodane diterpene in germander (*Teucrium chamaedrys*), is a structural analog of DB. Oral administration of teucrin A or extract from germander can induce significant hepatic necrosis in mice, which has been attributed to the metabolic activation of a furan moiety to 1,4-enedials reactive metabolites (Druckova and Marnett, 2006).

Available information on DB metabolism is insufficient. Thus, in the present study, DB metabolism in vitro was investigated using liver microsomes from humans and animals, such as rats, mice, rabbits, and guinea pigs, using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS). DB metabolites in urine, feces, bile, and plasma of rats after oral administration of DB were also identified. Glutathione (GSH) and methoxylamine were used as trapping agents in the incubation in vitro once the reactive metabolites were distinguished by the formation of Tris base conjugates of DB. To confirm

This work was supported by the Doctoral Fund of Ministry of Education of China [20133107110012] (awarded to C.W.).

dx.doi.org/10.1124/dmd.114.058222.

[§]This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: AQ4N, 1,4-bis-([2-(dimethylamino-*N*-oxide)ethyl]amino)-5,8-dihydroxy-anthracene-9,10-dione; DB, diosbulbin B; DMDO, dimethyldioxirane; DMSO, dimethylsulfoxide; GSH, glutathione; HLM, human liver microsomes; HMBC, heteronuclear multiple-bond correlation; HPLC, high-performance liquid chromatography; IS, internal standard; L-739,010, (1*S*,5*R*)-3-cyano-1-(3-furyl)-6-[6-[3-(3 α -hydroxy-6,8-dioxabicyclo [3.2.1]octanyl)pyridine-2-yl-methoxy]naphthalene; MGCD, mono-GSH conjugates of DB; P450, cytochrome P450; Q-TOF, quadrupole-time-of-flight; RAF, relative activity factor; RLM, rat liver microsomes; UPLC-MS/MS, ultra-performance liquid chromatography–tandem mass spectrometry.

the structures of the reactive metabolites, mono-GSH conjugates of DB (MGCD) were chemically synthesized, purified by semipreparative high-performance liquid chromatography (HPLC), and identified by NMR spectroscopy. The bioactivation enzymes of DB were identified using human recombinant cytochrome P450 (P450) enzymes. Michaelis-Menten constants K_m and V_{max} were estimated by the formation of MGCD. Moreover, the importance of P450 isoforms for the bioactivation of DB was determined using specific inhibitory studies and relative activity factor (RAF) approach for pooled human liver microsomes (HLM). This study could contribute to the understanding of the pharmacological activity and hepatotoxicity of DB.

Materials and Methods

Chemicals and Reagents

DB (>98% purity) was purchased from Shanghai Forever Biologic Technology Co., Ltd. (Shanghai, China). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, NADPH, Tris base, reduced GSH, methoxylamine, sulfaphenazole, and (S)-(+)-N-3-benzylirivanol were from Sigma-Aldrich (St. Louis, MO). Human enzymes CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, and human P450-reductase coexpressed in *Escherichia coli*, supplemented with purified cytochrome b_5 , were from Shanghai Ruide Institute of Liver Disease (Shanghai, China). Pooled HLM of 32 humans (with equal numbers of males and females) were from BD Biosciences (Woburn, MA). Ketoconazole and jatrorrhizine hydrochloride [internal standard (IS)] were purchased from the National Institute for Food and Drug Control (Beijing, China). Ultrapure water (>18 m Ω) was obtained from a Milli-Q water purification system (Millipore, Billerica, MA). HPLC-grade acetonitrile was purchased

from Fisher Scientific (Fair Lawn, NJ). NMR solvents used were 99.8% ampoule dimethylsulfoxide (DMSO)- d_6 or pyridine- d_5 (Cambridge Isotope Laboratories Inc., Tewksbury, MA). All other chemicals used were of analytical grade.

Animals. Male Sprague-Dawley rats (weighing 200–250 g) were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (Shanghai, China). All experiments performed in rats were in accordance with the People's Republic of China legislation on the use and care of laboratory animals and were approved by the Experimental Animal Ethical Committee, Shanghai University of Traditional Chinese Medicine.

Preparation of Liver Microsomes

Liver microsomes were prepared from Sprague-Dawley rats, mice, rabbits, and guinea pigs according to the reported method (Lu and Levin, 1972). The method used uninduced animals as well as animals that were pretreated with phenobarbital (50 mg/kg i.p. for 3 days). Protein concentrations were determined according to the method described by Bradford (1976).

Microsomal Incubations

The incubation system (100 μ l) contained DB (100 μ M), microsomal proteins (1 mg/ml), and a NADPH-regenerating system that included NADPH (1 mM), glucose 6-phosphate (10 mM), and glucose-6-phosphate dehydrogenase (1 unit/ml) in buffer A (50 mM Tris-HCl, pH 7.4, containing 4 mM MgCl₂) or buffer B (100 mM phosphate buffer, pH 7.4, containing 4 mM MgCl₂). DB was dissolved in acetonitrile, and the final concentration of acetonitrile in the incubation system was <1%. The system was preincubated for 5 minutes at 37°C and initiated by adding NADPH. Control incubations were conducted without DB or NADPH. Incubations were stopped after 1 hour by the addition of 500 μ l

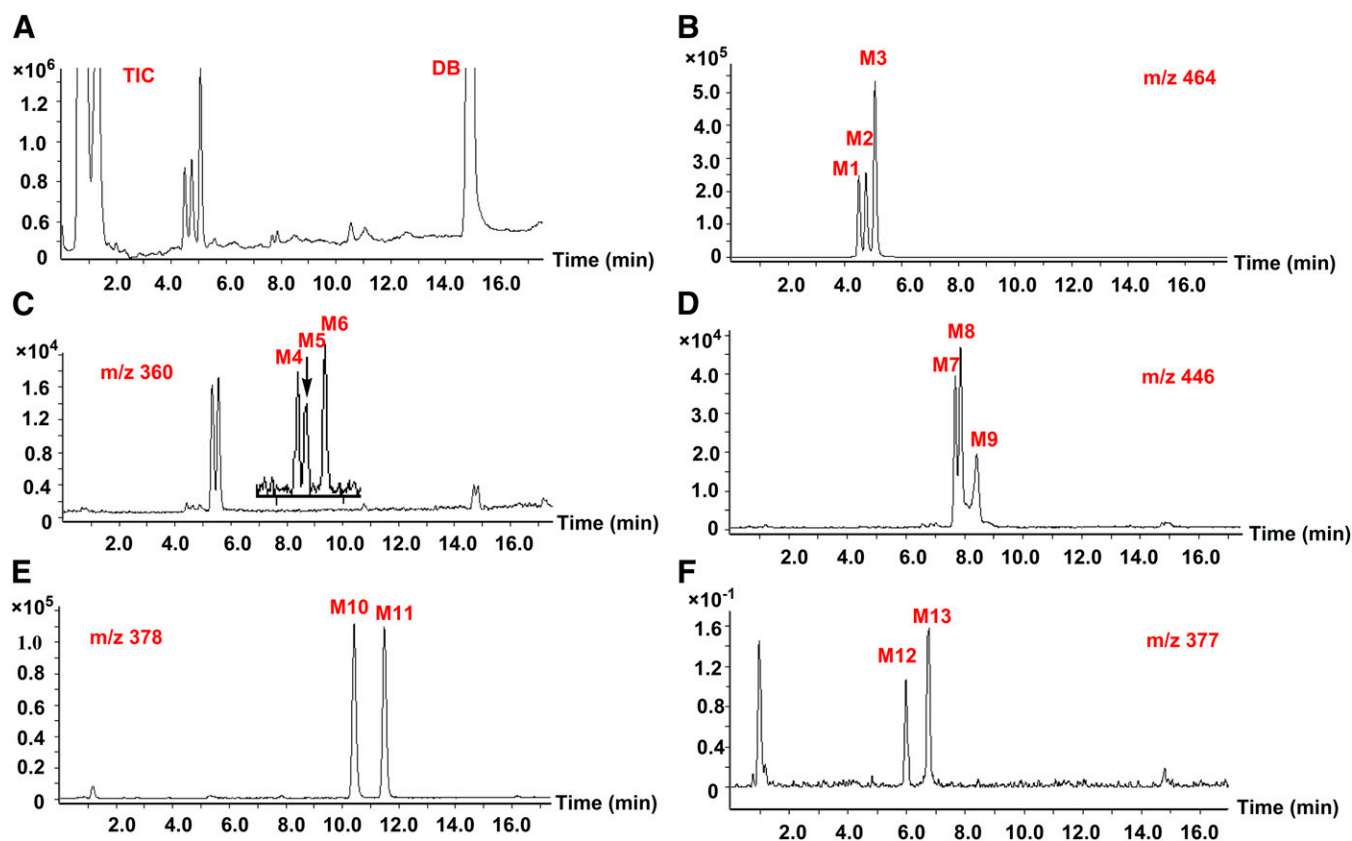


Fig. 1. The typical total ion current (TIC) (A) and extract ion spectra of m/z 464 (B), m/z 360 (C), m/z 446 (D), m/z 378 (E), and m/z 377 (F) from microsomal metabolites of diosbulbin B in Tris-HCl buffer (M1 to M11) and phosphate buffer (M12 and M13), using an Agilent 6410 Triple Quad LC-MS/MS system with eluting program A.

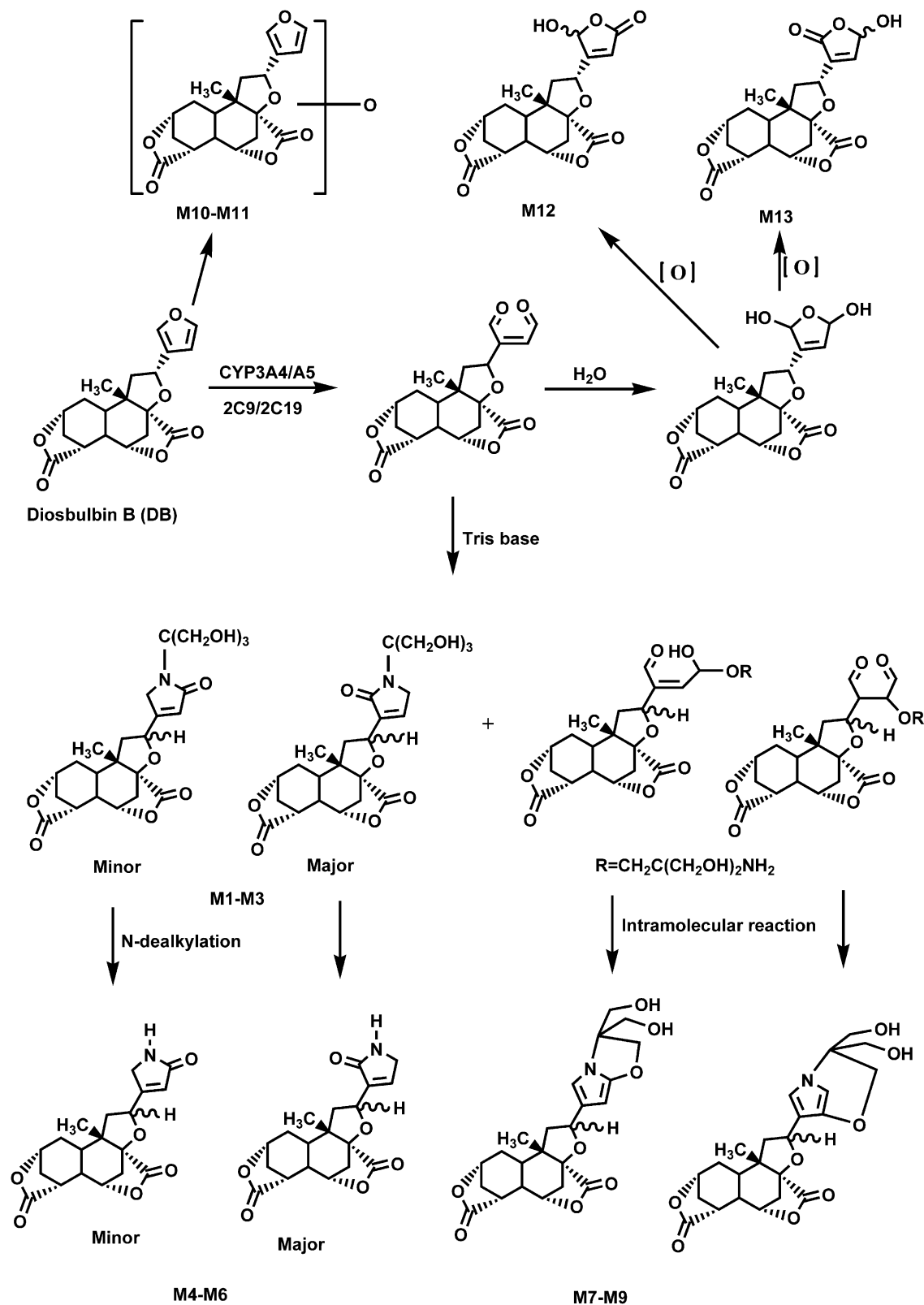


Fig. 2. Metabolic profile of diosbulbin B in vitro in Tris-HCl buffer (M1 to M11) and phosphate buffer (M12 and M13).

of ice-cold acetonitrile and then vortex-mixed for 1 minute. Denatured proteins were separated by centrifugation at 16,000g for 10 minutes. The supernatant (540 μ l) was evaporated to dryness under a nitrogen stream, reconstituted using 90 μ l of 10% acetonitrile, vortex-mixed for 1 minute, and centrifuged at 16,000g at 4°C for 10 minutes. The supernatant (5 μ l) was injected into the

UPLC-MS/MS system for analysis (see *UPLC-MS/MS Analysis to Screen Metabolites In Vitro and In Vivo (Analysis A)*).

Trapping experiments were conducted in the presence of nucleophiles, including GSH and methoxylamine (each at 10 mM), and the samples were prepared by the aforementioned procedure.

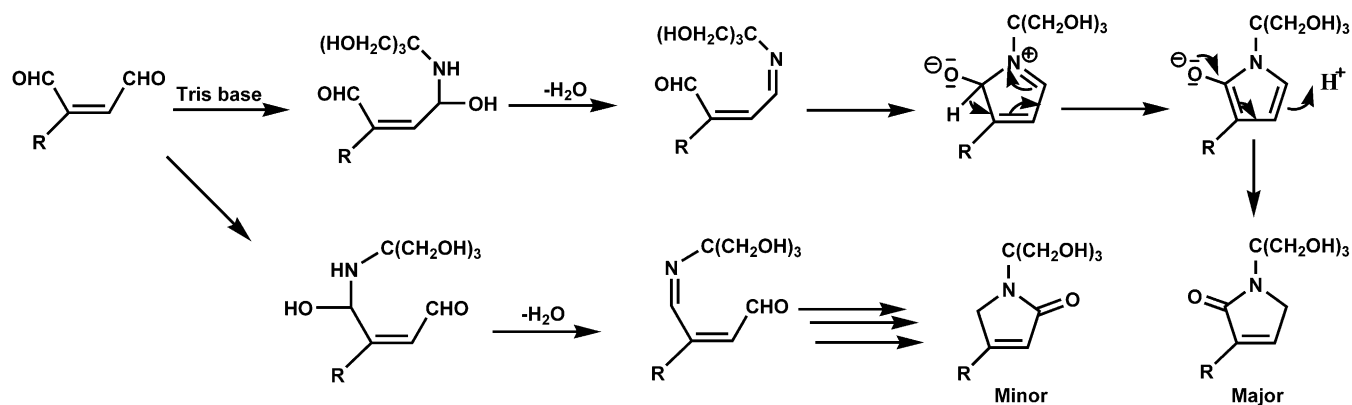


Fig. 3. Proposed mechanisms for the formation of Tris base conjugates with the enedial derivatives of DB (M1 to M3).

Metabolism In Vivo in Rats

Investigation of Metabolites in Plasma of Rats. Male rats were fasted for 12 hours and had free access to water prior to the experiments. Blank blood samples were drawn from the retro-orbital plexus before administration. Then DB formulated in 0.5% methylcellulose suspension was orally administered by gavage to rats in single doses of 32, 75, and 150 mg/kg. Blood samples (~300 μ l) were drawn at 0.5, 1, 2, 4, 8, 12, and 24 hours after DB administration. Plasma was generated by centrifugation at 10,000g for 5 minutes. Acetonitrile (5 ml) was added to the combined plasma (1 ml) to precipitate the protein, then vortex-mixed for 1 minute and centrifuged at 16,000g for 10 minutes. The supernatant was evaporated to dryness under nitrogen stream, reconstituted using 200 μ l of 10% acetonitrile, vortex-mixed for 1 minute, and centrifuged at 16,000g at 4°C for 10 minutes. The supernatant (5 μ l) was injected into the UPLC-MS/MS system for analysis (see *UPLC-MS/MS Analysis to Screen Metabolites In Vitro and In Vivo (Analysis A)*).

Investigation of Metabolites in Urine and Feces of Rats. Rats were treated as described earlier. Blank urine, feces before dosing, and urine and feces from 0 to 24 hours after dosing were collected and stored at -80°C until analysis. Urine (1 ml) was processed using the procedure used to process the plasma. Feces were moistened with little water and then homogenized. All homogenized feces were extracted using 6-fold acetonitrile by ultrasonic wave for 1 hour and then centrifuged (Yang et al., 2013). The supernatant (100 μ l) was diluted with 100 μ l of water, and a 5- μ l aliquot of the diluted solution was injected into the UPLC-MS/MS system for analysis (see *UPLC-MS/MS Analysis to Screen Metabolites In Vitro and In Vivo (Analysis A)*).

Investigation of Biliary Metabolites in Rats. Rats were terminally anesthetized with urethane (1.4 g/ml in isotonic saline; 1.0 ml/kg intraperitoneally)

and cannulated via the bile duct. DB was suspended in 0.5% methylcellulose and injected into the stomach in a single dose of 75 mg/kg. Drug-free bile was collected for 30 minutes before DB administration. Bile was then collected from 0 to 4 hours and 4 to 8 hours after administration. Bile samples were prepared similar to the plasma and urine samples as described earlier.

UPLC-MS/MS Analysis to Screen Metabolites In Vitro and In Vivo (Analysis A). Samples from incubation and biologic specimens (plasma, bile, urine, and feces) were analyzed on an Agilent 6410 Triple Quad liquid chromatography-tandem mass spectrometry system (Agilent Technologies Inc., Santa Clara, CA) or a Waters ACQUITY UPLC-Micromass quadrupole-time-of-flight (Q-TOF) system (Waters, Milford, MA) using an Acquity UPLC BEH C₁₈ column (50 \times 2.1 mm, 1.7 μ m; Waters) at 40°C. The analytes were separated by a gradient mobile phase consisting of acetonitrile (A) and an aqueous solution of 0.1% formic acid (B) at a flow rate of 0.2 ml/min. The gradient elution program was as follows: 0–15.0 minutes, 5–25% A; 15.0–16.0 minutes, 15–80% A; 16.0–17.0 minutes, 80–5% A (program A) or 0–5.0 minutes, 10–20% A; 5.0–12.0 minutes, 20–27% A; 12.0–15.0 minutes, 27–95%; 15.0–17.0 minutes, 95–5% A (program B). The temperature of the autosampler was maintained at 3–6°C. MS was operated in positive mode under the following operating Triple Quad parameters: gas temperature, 325°C; gas flow, 10 l/min; nebulizer, 35 psi; capillary voltage, 4.0 kV; fragmentor, 130 V; cell accelerator, 3 V; and dwell, 200 ms. For Q-TOF, the parameters were as follows: capillary voltage, 3.0 kV; sampling cones, 20 V; extraction cone, 4.0 V; source temperature, 120°C; desolvation temperature, 350°C; cone gas, 50 L/h; and desolvation gas, 600 L/h.

Elemental composition calculations for the exact masses were performed off-line using MassLynx V4.1 (Waters), which was used to apply the spectrum that was manually generated for every peak. Potential assignments were calculated

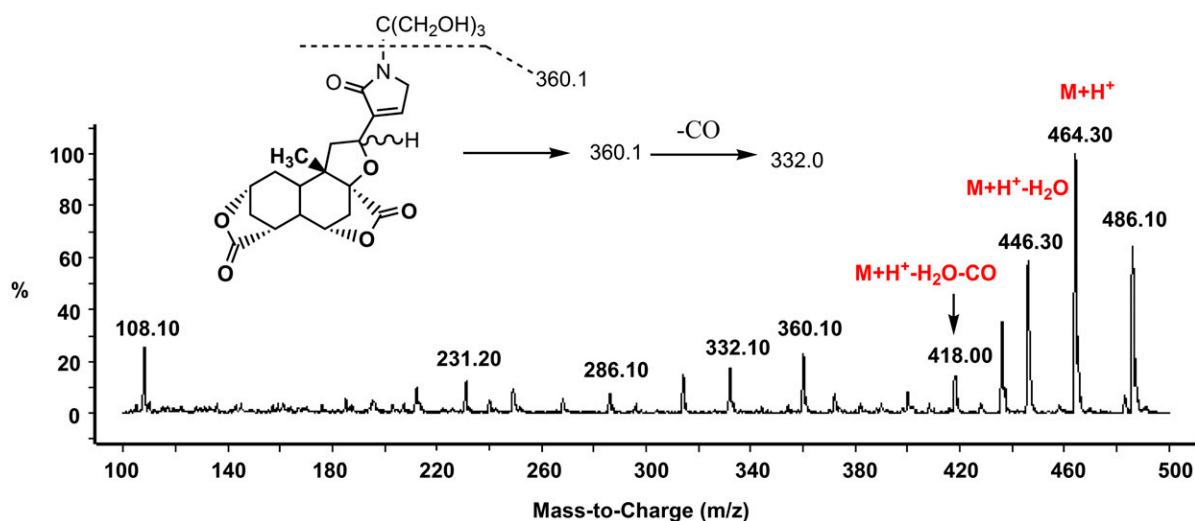


Fig. 4. Structural formula of M3, including its fragmentation, as shown by its mass spectrum using an Agilent 6410 Triple Quad LC-MS/MS system.

TABLE 1
Mass spectral data for the metabolites and diosbulbin B

Compound	<i>m/z</i>	MS ²	HRMS (Measured)	HRMS (Calculated)	Chemical Formula	Error
Diosbulbin B	362.2 (M+NH ₄ ⁺)	317.2, 299.3, 271.4, 253.3	362.1618	362.1604	C ₁₉ H ₂₀ O ₆	<i>ppm</i> −3.9
M1–M3	464.2 (M+H ⁺)	446.3, 428.3, 418.0, 400.1, 372.3, 360.3, 332.1	464.1927–464.1939	464.1921	C ₂₃ H ₂₉ NO ₉	−3.9 ^a
M4–M6	360.1 (M+H ⁺)	332.0, 314.2, 286.0,	360.1438–360.1440	360.1447	C ₁₉ H ₂₁ NO ₆	1.9 ^a
M7–M9	446.2 (M+H ⁺)		446.1790–446.1797	446.1815	C ₂₃ H ₂₇ NO ₈	5.6 ^a
M10–M11	378.2 (M+NH ₄ ⁺)	333.3, 315.1, 287.2	378.1553, 378.1537	378.1553	C ₁₉ H ₂₀ O ₇	4.2 ^a
M12–M13	377.2 (M+H ⁺)	359.1, 331.1, 303.1, 274.9, 257.1, 228.9	377.1232, 377.1239	377.1236	C ₁₉ H ₂₀ O ₈	1.1 ^a
M14–M16	360.2 (M+H ⁺)	332.2, 314.2	360.1425–360.1525	360.1447	C ₁₉ H ₂₁ NO ₆	6.1 ^a
M17–M18	390.2 (M+H ⁺)	362.2, 344.2	390.1562, 390.1534	390.1553	C ₂₀ H ₂₃ NO ₇	−2.3 ^a
M19–M20	390.2 (M+H ⁺)	362.2, 330.2, 312.1	390.1561, 390.1544	390.1553	C ₂₀ H ₂₃ NO ₇	2.3 ^a
M21–M22	392.2 (M+H ⁺)	374.2, 343.2	392.1718, 392.1737	392.1709	C ₂₀ H ₂₅ NO ₇	−7.1 ^a
M23–M26	419.2 (M+H ⁺)	388.2, 387.2, 359.2, 349.1, 341.2, 309.1	419.1820–419.1838	419.1818	C ₂₁ H ₂₆ N ₂ O ₇	−4.8 ^a
M27–M31	632.2 (M+H ⁺)	557.2, 335.6 (M+K ⁺ +H ⁺)	632.1899–632.1933	632.1914	C ₂₉ H ₃₃ N ₃ O ₁₁ S	−3.5 ^a
M32–M34	650.2 (M+H ⁺)	632.2, 575.2	650.1993–650.2010	650.2020	C ₂₉ H ₃₅ N ₃ O ₁₂ S	1.5 ^a
M35	939.3 (M+H ⁺)	864.2, 789.2, 761.2, 470.1 (M+2H ⁺)	939.2762	939.2752	C ₃₉ H ₅₀ O ₁₇ N ₆ S ₂	−1.1

HRMS, High Resolution Mass Spectrometry.
^aThe absolute value of the error is the biggest.

using monoisotopic masses with a tolerance of 10 ppm deviation. The number and types of expected atoms were set as follows: carbons ≤ 60, hydrogens ≤ 120, oxygens ≤ 30, nitrogens ≤ 30, and sulfurs ≤ 5.

Chemical Synthesis of Main Metabolites (GSH Conjugates of DB)

Dimethyldioxirane. Dimethyldioxirane (DMDO) was prepared according to the reported method (Murray and Singh, 1997) with a slightly modified generator apparatus in which another Dewar condenser was connected to the receiving flask instead of the air condenser. In brief, peroxymonosulfate (oxone, 50 g, 0.0813 mol) was added in portions to a stirred mixture of water (20 ml), acetone (12 ml, 0.163 mol), and sodium bicarbonate (24 g) under nitrogen atmosphere. An acetone-water (14 ml, 20ml) mixture was simultaneously added dropwise. The reaction proceeded for 15 minutes, and a slight vacuum was applied to the reaction assembly. The distillate was collected for 20 minutes in the receiving flask containing Na₂SO₄ and cooled in a dry ice/acetone bath. The receiving flask was also connected to a trap with potassium iodide solution. The distillate solution (3–4 ml), which typically affords DMDO concentrations of 0.02–0.04 M, was transferred to a dry ice bath and used immediately for the next step.

Enedial Derivative of DB. DB (10 mg, 0.029 mmol) was dissolved in 2 ml of acetone and precooled in a dry ice bath for 1 minute. Then 1.2 ml of DMDO was added immediately. The reaction mixture was allowed to warm up to room temperature and left to stand for 2 hours. A 200-μl aliquot of the reaction mixture was evaporated to dryness under nitrogen stream and reconstituted using 200 μl of 10% acetonitrile for UPLC-MS/MS analysis (see *UPLC-MS/MS Analysis to Screen Metabolites In Vitro and In Vivo (Analysis A)*) to confirm the disappearance of DB. The residual DMDO was bubbled off in the stream of nitrogen gas. The total volume was reduced to 0.5 ml and used immediately for the next step.

Reaction of Enedial Derivative of DB with GSH. GSH (17.8 mg, 0.058 mmol) was dissolved in 2 ml of phosphate buffer (pH 7.4), and a solution of enedial derivative of DB (0.5 ml) was added dropwise while shaking. The resulting solution was left to stand at room temperature for 15 minutes. A 10-μl aliquot of the solution was diluted with 1 ml of water and then subjected to the UPLC-MS/MS system (see *UPLC-MS/MS Analysis to Screen Metabolites In Vitro and In Vivo (Analysis A)*) to confirm the production of GSH adducts of DB. The final solution was stored at −80°C until the following separation.

Semipreparative HPLC Separation of Synthetic Products. The synthetic products were separated by a reverse phase-HPLC system using a DIKMA Spursil C18 column (250 × 10 mm, 5 μm; Dikma Technologies, Lake Forest, CA) at 25°C, with mobile phase consisting of 25% acetonitrile and 0.1% formic acid at the following gradient flow rates: from 0 to 15 minutes, 1.8 ml/min; and from 15 to 35 minutes, 1.6 ml/min. The wavelengths were set at 210 and 254 nm.

Incubations of DB with Recombinant Human P450 Enzymes

Recombinant human liver P450 isoenzymes of 100 pmol/ml (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, and 3A5) were incubated with 50 μM

DB in phosphate buffer solutions. The concentrations of GSH and the NADPH-regenerating system were similar to those in microsomal incubations. The incubations (100 μl), which were conducted in triplicate, were stopped by the addition of 500 μl of cold acetonitrile containing jatrorrhizine hydrochloride (IS) following the steps of the microsomal incubations. Microsomes from the same cell line that contain only the vector were used as control. The formation rate of metabolite M31, one of the main MGCDs, was determined by UPLC-MS/MS (see *UPLC-MS/MS Analysis for MGCD (M31) Formation (Analysis B)*).

Incubations of DB with Pooled HLM and Specific Chemical Inhibitors

To estimate the importance of P450 enzymes responsible for the bioactivation of DB, DB (60 μM) was incubated with pooled HLM (0.2 mg/ml) in the

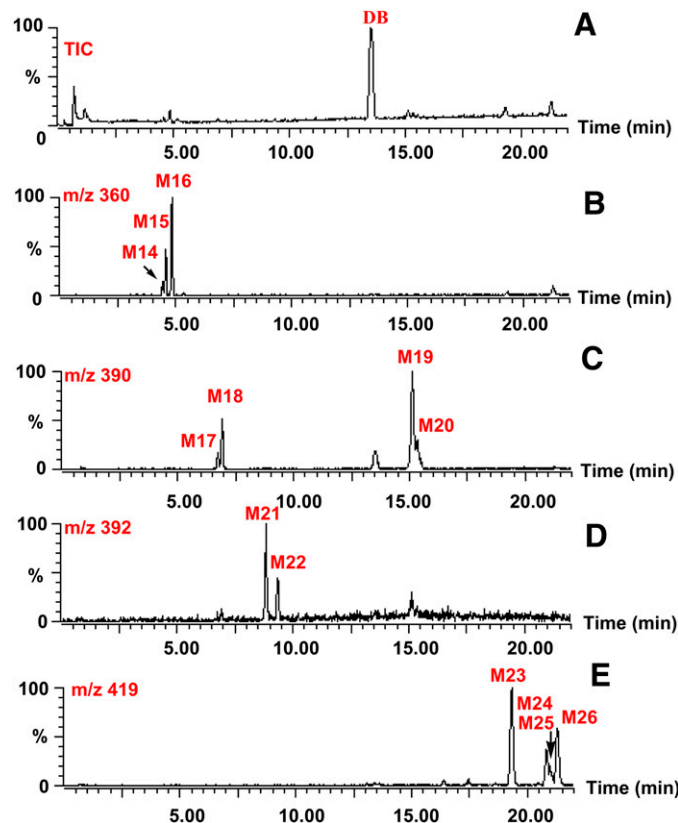


Fig. 5. The typical total ion current (TIC) (A) and extract ion spectra of *m/z* 360 (B), *m/z* 390 (C), *m/z* 392 (D), and *m/z* 419 (E) from microsomal metabolites of diosbulbin B trapped by methoxylamine in Tris-HCl buffer, using a Waters ACQUITY UPLC-Micromass Q-TOF system with eluting program A.

presence of a NADPH-regenerating system, GSH (10 mM), and chemical inhibitors at 37°C for 1 hour. The chemical inhibitors used were as follows: sulfaphenazole (10 μM) for CYP2C9, (*S*)-(+)-*N*-3-benzylrinivoranol (1 μM) for CYP2C19 (Walsky and Obach, 2003), and ketoconazole (1 μM) for CYP3A4/5 (Venkatakrishnan et al., 2001). Control incubations were conducted with vehicles but without chemical inhibitors. (*S*)-(+)-*N*-3-Benzylrinivoranol was dissolved in DMSO, sulfaphenazole in water, and ketoconazole in methanol. Organic solvents in the incubations were controlled within 1% of the incubation volume. Incubations in triplicate were processed as described for incubations with recombinant human P450 enzymes. Inhibition was calculated as the M31 formation rate in the inhibited ($v_{\text{inhibited}}$) samples ($n = 3$) relative to the control (v_{control}) samples ($n = 3$):

$$\text{inhibition} = (1 - v_{\text{inhibited}}/v_{\text{control}}) \times 100.$$

Enzyme Kinetics Studies on the Formation of MGCD (M31)

Incubations used to derive kinetic constants by the formation of M31 had the following DB and P450/HLM concentrations: DB, 5, 10, 20, 40,

60, 80, 100, 120, 160, 200, and 300 μM; CYP3A4, 10 pmol; CYP3A5, 15 pmol; CYP2C9, 25 pmol; CYP2C19, 15 pmol; and pooled HLM, 0.2 mg/ml. Incubation solutions (100 μl), which were conducted in triplicate, were transferred to an ice bath for 5 minutes after 1 hour at 37°C, followed by the sequential addition of 10 μl of acetonitrile containing jatrorrhizine hydrochloride (IS), 10 μl of DMSO, and 480 μl of acetonitrile. The following processing steps were similar to those of the microsomal incubations, except that the residual was reconstituted with 90 μl of aqueous solution containing 10% DMSO and 10% acetonitrile. The samples were analyzed by UPLC-MS/MS (see *UPLC-MS/MS Analysis for MGCD (M31) Formation (Analysis B)*). The enzyme kinetic investigations were performed within the range of linearity with respect to concentrations of P450 enzymes and DB, which were determined in preliminary experiments. Parent compound conversions were within 5% at all DB concentrations. Apparent K_m and V_{max} were estimated by non-linear regression using GraphPad Prism 5.0 (GraphPad Software Inc., San

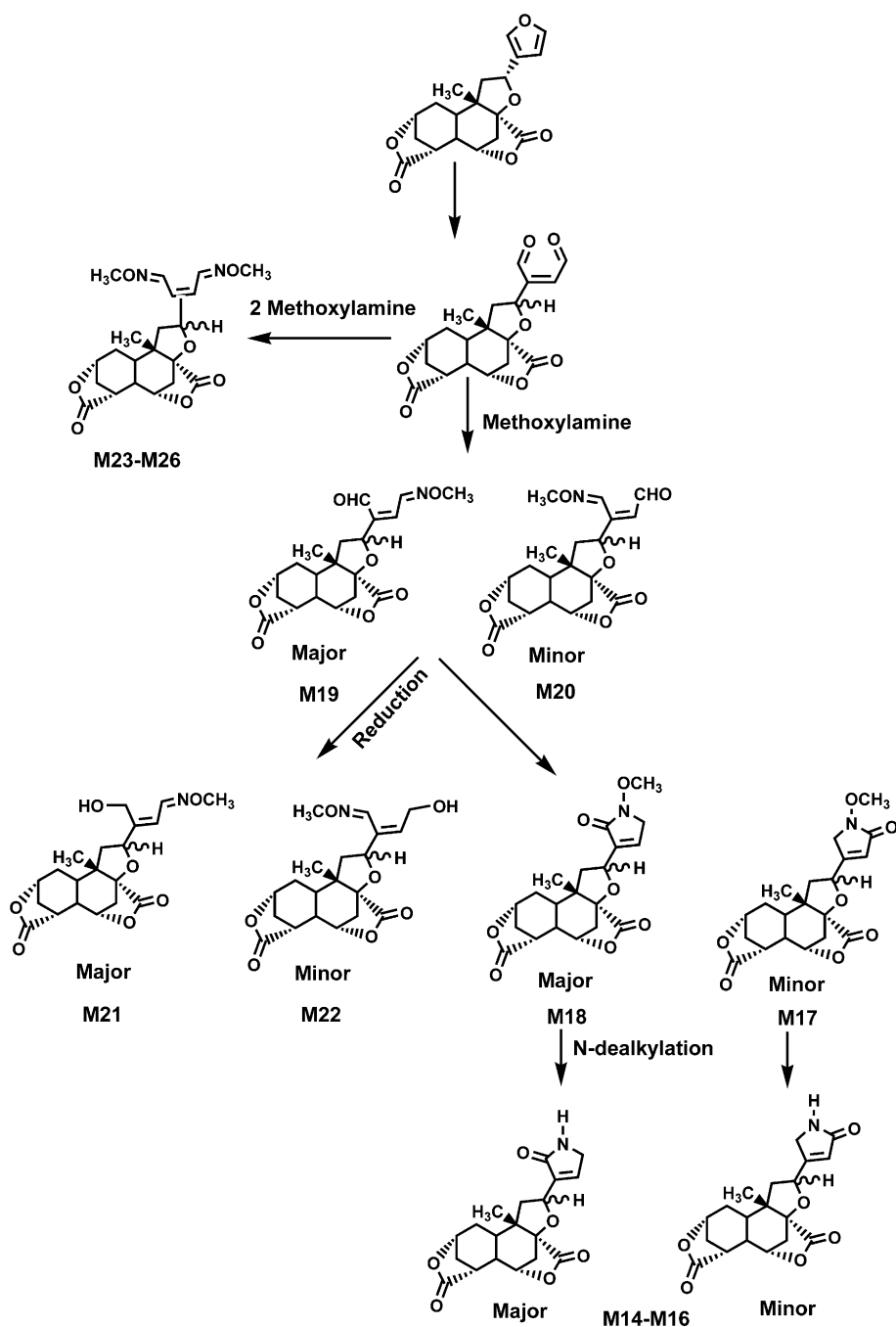


Fig. 6. Metabolic profile of diosbulbin B in vitro trapped by methoxylamine in Tris-HCl buffer.

Diego, CA). The data were fitted to the one-enzyme Michaelis-Menten equation:

$$V = V_{\max} \times S / (K_m + S),$$

or the Michaelis-Menten model with uncompetitive substrate inhibition (Venkatakrisnan et al., 2001):

$$V = V_{\max} \times S / (K_m + S + S^2 / K_s).$$

Eadie-Hofstee transformations were used when multiple enzymes were involved or Hill equation was more suitable.

Prediction of Relative Contributions for P450 Enzymes in HLM to MGCD (M31) Formation

The relative contribution of P450 isoforms in HLM to the formation of M31 was predicted using RAFs (Crespi and Miller, 1999) calculated as

$$f_i(\%) = \text{RAF}_{i v_i}(s) / \sum \text{RAF}_{i v_i}(s) \times 100,$$

where f_i is the relative contribution of a specific P450 isoform, RAF_i is the relative activity factor, and v_i is the reaction velocity of the P450 isoform at a specific DB concentration. v_i was calculated by the Michaelis-Menten equation using the estimated V_{\max} and K_m . RAF_i was calculated as the rate for a probe substrate in HLM divided by the rate for a probe substrate with cDNA expressed. The probe substrates were testosterone, tolbutamide, and (*S*)-mephenytoin for CYP3A4/5, CYP2C9, and CYP2C19, respectively.

UPLC-MS/MS Analysis for MGCD (M31) Formation (Analysis B)

Chemically synthesized M31 was used as a standard substance. The UPLC-MS/MS method was fully validated according to the Bioanalytical Method Validation Guidance for Industry enacted by the Food and Drug Administration (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM368107.pdf>). Analysis was performed on an Agilent 6410 Triple Quad LC-MS/MS system (Agilent Technologies Inc.) using an Acquity UPLC BEH C₁₈ column (50 × 2.1 mm, 1.7 μM; Waters) at 40°C. The analytes were separated by a gradient mobile phase consisting of acetonitrile (A) and an aqueous solution of 0.1% formic acid and 5 mM ammonium acetate (B) at a flow rate of 0.3 ml/min. The gradient elution program was as follows: 0–4.0 minutes, 10–16.4% A; 4.0–6.0 minutes, 16.4–40% A; 6.0–8.0 minutes, 40–80% A; and 8.0–10.0 minutes, 80–10% A. The temperature of the autosampler was maintained at 4°C. The injection volume was 5 μl. The tandem mass spectrometry system was operated in positive mode under the following operating parameters: gas temperature, 325°C; gas flow, 10 l/min; nebulizer, 35 psi; capillary voltage, 4.0 kV; fragmentor, 190 V for M31 and 170 V for IS; cell accelerator, 3 V for M31 and 1V for IS; and dwell, 200 ms. Quantification transitions for multiple reaction mode were set at m/z 632.1 → m/z 557.2 for M31 and m/z 230.1 → m/z 120.9 for IS, with collision energy set to 20 V for both substances. The data were processed using the Agilent MassHunter Qualitative Analysis B.04.00 Software (Agilent Technologies Inc.). Calibration standards with seven levels (0.083, 0.166, 0.332, 0.83, 1.66, 3.32, and 6.64 μM) were prepared daily by adding stock solution of M31 (86 μM) by serial dilutions. Calibration standards were analyzed in triplicate, and quality control samples at three levels (0.083, 0.332, and 3.32 μM) were run daily and checked to confirm that they were at nominal value.

Results

Identification of Metabolites in Microsomal Incubations

Thirteen metabolites dependent on the NADPH-regenerating system were detected in the microsomal incubations (Fig. 1). These metabolites included 11 metabolites in buffer A and two in buffer B.

M1 to M3. Metabolites M1 to M3 were observed at m/z 464.2 ($M + H^+$), with a chemical formula of C₂₃H₂₉NO₉ by Q-TOF mass, which indicated that one molecule of Tris base (C₄H₁₁NO₃) might be conjugated to DB. M1 to M3 were identified as Tris base conjugates of DB

formed via reactive metabolites (Fig. 2) with a proposed mechanism (Fig. 3) similar to the bioactivation of teucin A (Druckova and Marnett, 2006). The furan moiety of DB was catalyzed by P450 to form reactive metabolites, 2-butene-1,4-dial derivatives of DB, and one of the aldehyde groups of the reactive metabolites reacted with the amine group of Tris base to form the Schiff base. The Schiff base then reacted with the other aldehyde group of the reactive metabolite via addition reaction, and intramolecular rearrangement occurred to form substituted pyrrol derivatives. Signals of fragments with loss of H₂O, CO, or C (CH₂OH)₃ were observed (Fig. 4; Table 1), supporting the deduced structures of M1 to M3. According to the synthesis of the conjugates of teucin A with *N*-acetyl lysine (Druckova and Marnett, 2006), the configuration of C12 in DB could be changed in the metabolites. The 3-substituted pyrroline-2-one could be the major metabolites (M3), similar to the main conjugates of teucin A with *N*-acetyl lysine. M1 to M3 might be composed of four compounds which have two structures, 3-substituted pyrroline-2-one and 4-substituted pyrroline-2-one, with different configurations in C12 (Fig. 2). However, it is difficult to distinguish each one from mass data except the major metabolites (M3).

M4 to M6. Metabolites M4 to M6 exhibited a signal at m/z 360.1 ($M + H^+$), 15 Da higher than DB in molecular weight. Signals of fragments m/z 332.0 ($M + H^+ - CO$), m/z 314.2 ($M + H^+ - CO - H_2O$), and m/z 286.0 ($M + H^+ - CO - H_2O - CO$) were observed, which demonstrated similar DB fragmentation. From their chemical formula (C₁₉H₂₁NO₆), metabolites M4 to M6 were deduced as the *N*-dealkylated products of M1 to M3 with a loss of C (CH₂OH)₃ (Fig. 2).

M7 to M9. Metabolites M7 to M9 were observed at m/z 446.2 ($M + H^+$), 18 Da lower than that of M1 to M3, suggesting that an intramolecular reaction might occur when Tris base is conjugated to the reactive metabolites of DB (Fig. 2). This reaction is probably similar to the formation of M27 to M31 (see *M27 to M31*). However, no fragment signals were observed, which resulted in the deduction with slight uncertainty.

M10 and M11. Metabolites M10 and M11 showed signals at m/z 378.2 ($M + NH_4^+$), with a similar parent ion of DB (m/z 362.2, $M + NH_4^+$) and similar DB fragmentation patterns (Table 1). Thus, M10 to M11 were identified as the mono-oxydates of DB.

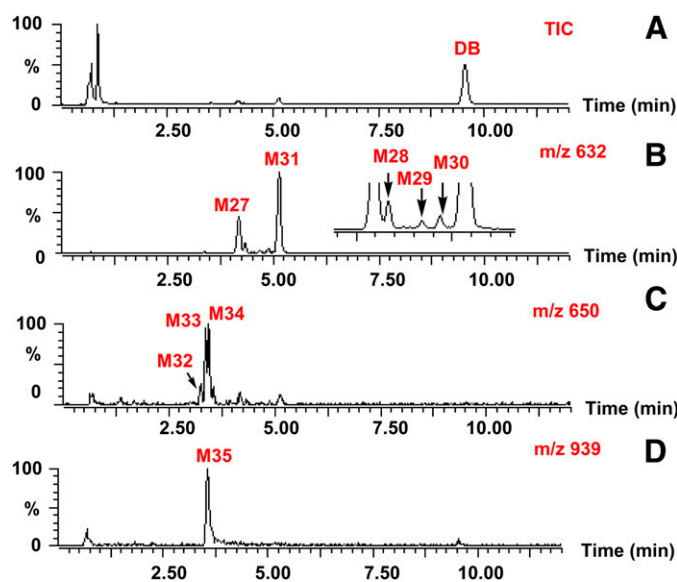


Fig. 7. The typical total ion current (TIC) (A) and extract ion spectra of m/z 632 (B), m/z 650 (C), and m/z 939 (D) from microsomal metabolites of diosbulbin B trapped by GSH, using a Waters ACQUITY UPLC-Micromass Q-TOF system with eluting program B.

M12 and M13. Microsomal incubations of DB in buffer B (phosphate buffer) generated two other products, M12 and M13 (m/z 377.2, $M + H^+$), in addition to M10 and M11, with the disappearance of M1 to M9 when buffer A (Tris buffer) was replaced. M12 and M13 were also involved in the formation of reactive metabolite and were identified as hemiacetal lactones (see *Identification of the Synthesized Products*). The 2-butene-1,4-dial moiety of the reactive metabolite turned into hemiacetal and was oxidized to hemiacetal lactones (Fig. 2).

M1 to M13 were all observed in the incubations with liver microsomes of rabbits and guinea pigs, and M11 was absent in the microsomal incubations of rats, mice, and humans.

Trapping experiments using methoxylamine in buffer A with rat liver microsomes (RLM) resulted in a series of metabolites (M14 to M26; Fig. 5) along with the disappearance of M1 to M9.

M14 to M16. Metabolites M14 to M16 were observed at m/z 360.2 ($M + H^+$), with chemical formulae ($C_{19}H_{21}NO_6$) and fragments (Table 1) similar to M4 to M6, as well as similar DB relative retention times. M14 to M16 were identified as *N*-dealkylated products of conjugates of methoxylamine and DB (Fig. 6), consistent with M4 to M6.

M17 to M20. Metabolites M17 to M18 and M19 to M20 had the same parent ion signals at m/z 390.2 ($M + H^+$), with different fragment signals. Fragments with loss of CH_3OH (32 Da) were found in the MS spectra of M19 and M20, but were absent in M17 and M18, indicating that they had different structures. M17 and M18 were assigned as 3-substituted or 4-substituted *N*-methoxy pyrroline-2-one, which was deduced from their fragmentation pattern similar to that of M14 to M16. According to the NMR analysis of similar metabolites, L-739,010

[(1*S*,5*R*)-3-cyano-1-(3-furyl)-6-{6-[3-(3 α -hydroxy-6,8-dioxabicyclo[3.2.1]octanyl)]pyridine-2-yl-methoxyl}naphthalene] (Zhang et al., 1996), M19, and M20 were deduced as a 1-*O*-methyloxime-2-butene 4-aldehyde derivative of DB (Fig. 6), and the double bond between carbon and nitrogen in the structures could turn into a triple bond after losing CH_3OH . Their reduction products, M21 and M22, also supported the deduced structures. M19 and M20 could transform into M17 and M18 by intramolecular reaction.

M21 and M22. Metabolites M21 and M22 had an m/z 2 Da higher than M19 and M20 and were eluted earlier than M19 and M20, suggesting they were reduction products of M19 and M20. Thus, M21 and M22 were deduced as 1-*O*-methyloxime-2-butene 4-alcohol derivatives of DB.

M23 to M26. Metabolites M23 to M26 were observed at m/z 419.2 ($M + H^+$), 29 Da higher than that of M19 and M20. The chemical composition ($C_{21}H_{26}N_2O_7$) of M23 to M26 indicated that two molecules of methoxylamine were conjugated to DB. Fragmentation with loss of one molecule of CH_3OH (32 Da) from m/z 419.2 to m/z 387.2 suggested that M23 to M26 might have structures similar to M19 and M20. M23 to M26 were identified as the bis-*O*-methyloxime derivatives of DB, supported by their fragmentation with loss of another molecule of CH_3OH (32 Da) from m/z 341.2 to m/z 309.1 (Table 1).

The same GSH adducts (M27 to M35; Fig. 7) were observed in other trapping experiments with GSH as a capture reagent in buffer A or buffer B with RLM.

M27 to M31. The major metabolites M27 to M31 exhibited an m/z 632.2 ($M + H^+$) with a chemical composition of $C_{29}H_{33}N_3O_{11}S$, which

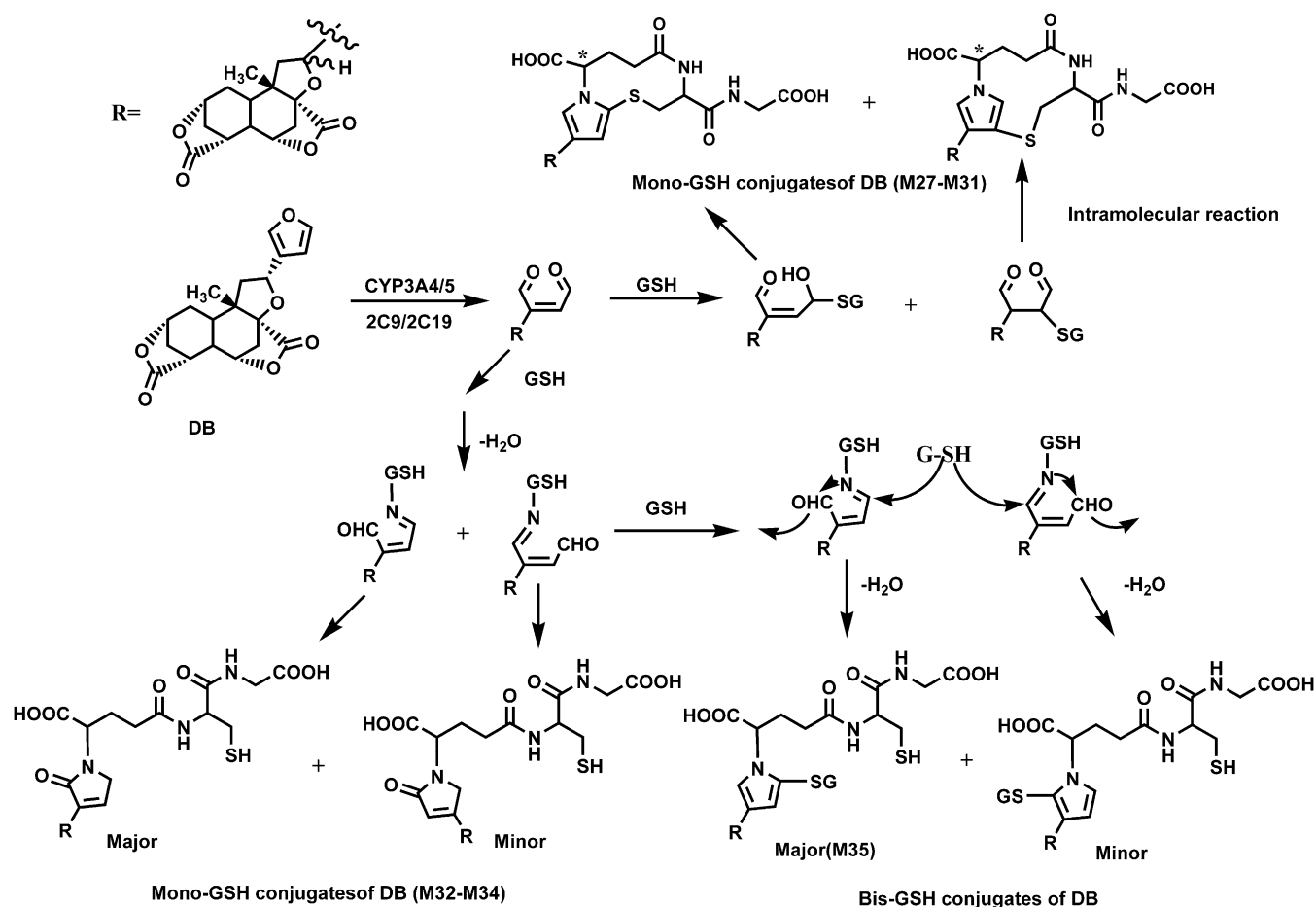


Fig. 8. Metabolic profile of diosbulbin B trapped by GSH.

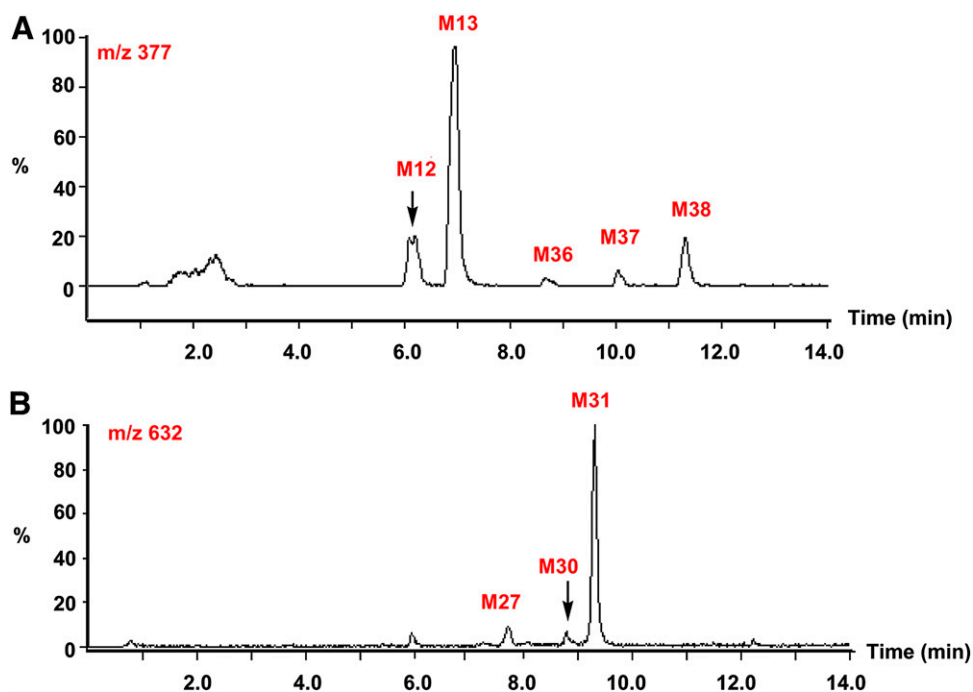


Fig. 9. Metabolites found in the feces of rats that were orally administered DB at 32 mg/kg using an Agilent 6410 Triple Quad LC-MS/MS system with eluting program A (A) and 150 mg/kg using a Waters ACQUITY UPLC-Micromass Q-TOF system with eluting program B (B).

indicated that one molecule of GSH conjugated to DB and was supported by the fragment signal (m/z 557.2) with loss of glycine. These metabolites were generated by intramolecular reaction after GSH conjugated to the 2-butene-1,4-dial derivatives of DB (Lu et al., 2009) (Fig. 8). The main metabolite, M31, was composed of 3-mercapto-pyrrol (M31a) and 2-mercapto-pyrrol derivatives of DB (M31b). Similar to M1 to M3, the configuration of C12 could be changed in GSH conjugates of DB. Configurations of chiral carbons in GSH moieties also could be changed (see *Identification of the Synthesized Products*). Therefore, M27 to M30 might be composed of the isomers of M31a and M31b with configurations changed in C12 or in the GSH moiety.

M32 to M34. The metabolites M32 to M34 with signals at m/z 650.2 ($M + H^+$) were generated by GSH conjugated to DB, similar to the formation of M1 to M3 (Fig. 8). These metabolites are supported by their chemical formula ($C_{29}H_{35}N_3O_{12}S$) and the fragment signal (m/z 575.2) with loss of glycine.

M35. A minor metabolite M35 was observed at m/z 939.2 ($M + H^+$). Its chemical formula ($C_{39}H_{50}O_{17}N_6S_2$), as well as fragment signals with loss of one glycine (m/z 864.2) and two glycines (m/z 789.2) from the parent molecule, indicated that two molecules of GSH were conjugated to DB. According to the formation of bis-GSH adducts of furan that were chemically synthesized (Chen et al., 1997), M35 was

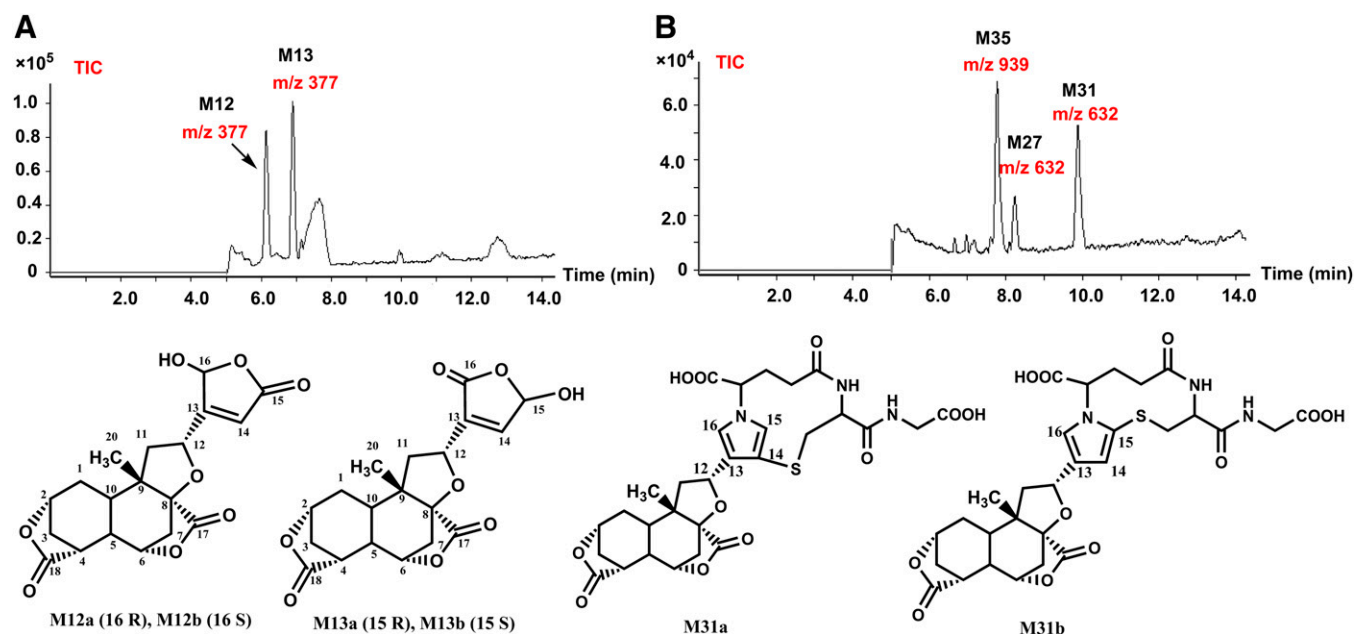


Fig. 10. The typical total ion current (TIC) spectra of the chemically synthesized products M12 to M13 (A), MGCD, and M31 (B), using an Agilent 6410 Triple Quad LC-MS/MS system with eluting program A, and their structures.

identified as a bis-GSH conjugate of DB. This metabolite was formed by the mercapto group of one GSH added to the Schiff base formed by the other GSH added to the aldehyde group of the reactive metabolites (Fig. 8).

Identification of Metabolites of DB In Vivo in Rats

No metabolite but DB was found in the plasma of rats after oral administration at three dosages, compared with the blank plasma. A signal of mono-oxydate (M10) in the urine of rats was observed when DB was administered at 32 and 75 mg/kg. Several metabolites (M36 to M38) with signals at m/z 377($M + H^+$), in addition to M12 and M13, were observed in the feces of rats when DB was administered at 32 mg/kg (Fig. 9). M36 to M38, which could not be identified by mass data, might be the isomers of M12 and M13. Mono-GSH adducts (m/z 632, $M + H^+$), including M27, M30, and M31, were detected in the bile and feces of rats at DB dosages of 75 and 150 mg/kg.

Identification of the Synthesized Products

The reaction mixture, exposed to air or not, would result in different products before the residual DMDO was bubbled off in the stream of nitrogen gas (see *Enedial Derivative of DB*). M12 and M13 (m/z 377) were the main products if the reaction mixture was exposed to air for a few minutes before it was concentrated under nitrogen. Otherwise, GSH adducts (m/z 632 and 939) were primarily produced if the reaction mixture was kept from air as long as possible (Fig. 10). M27, M31, and M35 could be observed in the total ion current spectrum, whereas minor M28 to M30 could only be observed in the extract ion spectrum.

M12 and M13 (at a proportion of 5:1) were eluted from the column at 21.3 and 24.6 minutes, respectively. M12 and M13 were then lyophilized to afford white powder, and each was composed of two isomers. Most signals in the ^{13}C -NMR spectrum of M12 are similar to those of DB (Yonemitsu et al., 1993), except that the signals belonging to the furan moiety of DB change to those of one carbonyl of ester (δ 171.23 for M12a, δ 171.38 for M12b), one hemiacetal group (δ 99.49 for M12a, δ 98.50 for M12b), and one alkene group

(δ 169.99 and 117.31 for M12a; δ 169.38 and 118.29 for M12b). M12a and M12b were identified as hemiacetal lactones, with the hemiacetal group connected to the quaternary carbon of the alkene group. In M13a and M13b, the hemiacetal group (δ 99.00 for M13a and M13b) was linked to the methyne carbon of the alkene group (δ 135.70 and 147.46 for M13a; δ 137.15 and 147.33 for M13b). The configurations of C16 in M12a and M12b were determined to be *R* and *S* using the Chem3D Ultra 8.0 software (CambridgeSoft Corporation, Waltham, MA), according to the differences in the chemical shifts of H11. The configurations of C15 in M13a and M13b were elucidated to be *R* and *S* by the same method (Fig. 10). The chemical shifts of C and H of M12 and M13, attributed by the ^1H - ^1H correlation spectroscopy, heteronuclear single quantum coherence, and heteronuclear multiple-bond correlation (HMBC) spectra, are shown in Table 2.

M31 was eluted from the column at 28.6 minutes, and then lyophilized to afford white powder. Signals similar to those of DB in the ^{13}C -NMR spectrum were all doubled, which indicated that M31 is composed of two isomers (see Fig. 10; Supplemental Fig. 1). The proportions of M31a and M31b were estimated to be approximately 1.2:1 according to the peak area of similar ^1H -NMR signals. M31a is 3-mercapto substituted at the pyrrole moiety supported by the HMBC correlation between H12 (δ 5.36, dd) and the quaternary carbon (δ 107.11) (see Supplemental Fig. 2). M31b is 2-mercapto substituted at the pyrrole moiety because no similar HMBC correlation was observed. Correlations between H12 and H20 (CH_3) in the nuclear Overhauser effect spectroscopy of M31a and M31b suggested that the configurations of C12 of M31a and M31b were consistent with that of DB (see Supplemental Fig. 3). NMR data of M31a and M31b are shown in Table 3.

Another MGCD, an analog of M31, was eluted at 30.3 minutes and then lyophilized to afford white powder. The ^1H -NMR spectrum of this MGCD (data not shown) was very similar to that of M31. Correlations between H12 and H20 (CH_3) in the nuclear Overhauser effect spectroscopy spectrum were also observed, which suggests that the MGCD was composed of isomers of M31a and M31b with configurations changed in the GSH moiety. The ^{13}C -NMR

TABLE 2
 ^1H (600 MHz) and ^{13}C -NMR (150 MHz) data for M12a/M12b and M13a/M13b (pyridine- d_5)

C/H Number	M12a/M12b			M13a/M13b		
	δ_{H}	$J_{\text{H-H}}$ Values	δ_{C}	δ_{H}	$J_{\text{H-H}}$ Values	δ_{C}
C-1/H-1	1.50(m) 1.84(m)		29.52/29.92	1.23(m)/1.43(m) 1.71(m)/1.82(m)		29.68/29.22
C-2/H-2	4.76(dd)	5.5, 5.5	76.94	4.74(dd)	5.5, 5.5	76.74
C-3/H-3	1.72(d) 2.34(dd)	11.7 5.4, 11.7	39.20	1.67(d) 2.33(dd)	11.5 5.5, 11.5	39.00
C-4/H-4	2.79(d)	4.4	42.42	2.77(d)	5	42.19
C-5/H-5	1.99(d)	12.0	42.33	1.92(d)	11.7	42.07/41.98
C-6/H-6	4.78(d)	5.7	78.11/77.9	4.73(d)	6.0	77.66/77.60
C-7/H-7	2.13(d) 2.53(dd)	11.6 5.8, 11.6	37.19	2.07(d) 2.51(dd)/2.49(dd)	11.6 5.5, 11.6	37.12
C-8			90.02/90.22			90.05/89.96
C-9			45.73			45.55/45.47
C-10/H-10	2.50(m)		40.98	2.04(m)/1.92(m)		38.89
C-11/H-11	2.0(dd)/1.82(dd) 2.24(dd)/2.08(dd)	11.7, 12.1/11.4, 11.7 6.0, 12.1/5.3, 11.7	40.52/39.63	1.22(dd)/1.65(dd) 1.98(dd)/2.23(dd)	10.5, 11.5 5.5, 11.5	40.75/40.38
C-12/H-12	5.39(dd)/5.20(dd)	5.5, 10.5	76.55/76.83	5.20(dd)	5.5, 10.5	75.46/75.16
C-13			169.99/169.38			135.7/137.15
C-14	6.56(s)/6.79(s)		117.31/118.29	7.90(s)		147.46/147.33
C-15/H-15			171.38/171.23	6.52(s)/6.62(s)		99.00
C-16/H-16	6.46(s)/6.71(s)		99.49/98.5			170.82
C-17			177.08/176.75			176.77
C-18			176.03			175.88
C-20/3H	1.09(s)/1.06(s)		16.21/15.94	0.98(s)/0.99(s)		15.87/15.75

C/H, Carbon/Hydrogen; d, doublet; dd, doublet doublet; m, multiplet; s, singlet.

spectrum of the MGCD was not acquired because of insufficient compound.

Incubations of DB with Recombinant Human P450 Enzymes

CYP3A4 and 3A5, as well as 2C19 and 2C9, were capable of bioactivating DB to M31, with CYP3A4 having the highest catalytic activity (Fig. 11).

Incubations of DB with Pooled HLM and Specific Chemical Inhibitors

The inhibited reaction activities for the formation of M31 caused by ketoconazole, sulfaphenazole, and (*S*)-(+)-*N*-3-benzylirvanol were 87, 15, and 27%, respectively. The results indicated that CYP3A4 was the major enzyme responsible for the bioactivation of DB.

Enzyme Kinetics Studies on MGCD (M31) Formation

The best fit of data was obtained using the Michaelis-Menten equation for enzyme kinetics with pooled HLM (Fig. 12). Eadie-Hofstee transformations showed no evidence of two-enzyme kinetics. CYP3A4, 3A5, 2C9, and 2C19 also exhibited Michaelis-Menten kinetics with no evidence of uncompetitive substrate inhibition. The enzyme kinetics parameters are shown in Table 4.

Prediction of Relative Contributions for P450 Enzymes in HLM to MGCD (M31) Formation

The RAF predictions were made at 5.0 and 40 μ M DB. P3A5 was not included in the RAF calculations. Considering the low V_{max} and large K_m , as well as the low expression in HLM, the contribution of CYP3A5 was lower than that of CYP2C19. The relative contributions of CYP3A4 were approximately 83.3 and 81.3%. CYP2C9 contributed about 10.5 and 12.2%, which were higher than the contributions of CYP2C19 (6.2 and 6.6%).

Discussion

DB metabolism in vitro and in vivo in rats, as well as the human enzymes involved, was first investigated. The main metabolic pathway of DB was catalyzed by CYP3A4/5, 2C9, and 2C19 to generate the reactive metabolites, 2-butene-1,4-dial derivatives of DB, which were captured by nucleophiles such as Tris base, methoxylamine, and GSH or oxidized to hemiacetal lactones. Tris base conjugates of DB (M1 to M3) that were observed at the total ion current spectrum of microsomal incubations in the Tris-HCl buffer incubation system provided important information on the metabolism pathway of DB, compared with the few data on metabolites in the phosphate buffer system. Tris base was used as a buffer constituent and as a trapping agent for the reactive metabolites of DB because Tris base could react with aldehyde to form the Schiff base (Cannon and Davison, 1976; Niedernhofer et al., 1997). The metabolites of DB found in vivo in rats were consistent with those observed in RLM incubations in vitro. M10 was found in rat urine and MGCD in rat bile and feces, as well as M12 and M13 in rat feces, which indicated that DB was also transformed into 2-butene-1,4-dial derivatives in vivo. DB metabolism in the liver and intestine decreased the bioavailability of DB when the compound was orally administered. In addition, species metabolic difference was observed in the formation of mono-oxydates; the two mono-oxydates (M10 and M11) were found in rabbits and guinea pigs and one (M10) in rats, mice, and human microsomal incubations. The results might have been caused by the varying expression of flavin-containing mono-oxygenases among these species (data not shown).

TABLE 3

^1H (600 MHz) and ^{13}C -NMR (150 MHz) data for M31a and M31b (DMSO- d_6)

C/H Number	M31a/M31b		
	δ_{H}	$J_{\text{H,H}}$ Values	δ_{C}
C-1/H-1	1.67(m) 1.93(m)		29.23/29.33
C-2/H-2	4.82(dd)	5.2, 5.2	77.34
C-3/H-3	1.93(d) 2.4(dd)	11.3 5.4, 11.3	38.76
C-4/H-4	2.71(d)	4.6	41.98/41.90
C-5/H-5	2.19(d)	10.1	41.53/41.36
C-6/H-6	4.73(d)/4.75(d)	5.2	77.78/78.51
C-7/H-7	2.11(d)/2.21(d) 2.32(dd)/2.43(dd)	10.7 5.4, 10.7	37.51/37.33
C-8			89.53/90.08
C-9			45.77/45.71
C-10/H-10	1.67(m)	11.3, 11.7	39.23/39.10
C-11/H-11	1.94(dd) 2.00(dd)	5.4, 11.7 5.2, 10.4	41.24
C-12/H-12	5.36(dd)/5.28(dd)	5.2, 10.1	75.68/74.90
C-13			126.39/125.15
C-14/H-14	6.99(bs) for 3b		107.11/128.64
C-15/H-15	6.64(bs) for 3a		121.82/107.73
C-16/H-16	6.96(bs)/6.83(bs)		134.01/122.38
C-17			177.89
C-18			176.84
C-20/3H	1.22(s)		16.86/16.73
Glu (-COOH)			179.22
Glu α -CH	4.81(m)		61.75
Glu β -CH ₂	2.1-2.3(m)		29.33
Glu γ -CH ₂	2.1-2.3(m)		29.92
Glu (-CO-NH-)			
Cys α -CH	4.23(m)/4.01(m)		58.16/56.45
Cys β -CH ₂	2.97(m)/3.21(m) 2.58(m)/2.81(m)		35.9/33.57
Cys (-CO-NH-)			171.26/171.40
Gly CH ₂	3.62 (overlapped)		41.77
Gly (-COOH)			171.26/171.40

bs, broad singlet; C/H, Carbon/Hydrogen; d, doublet; dd, doublet doublet; m, multiplet.

MGCD was finally synthesized because of the extremely low yield of metabolites in microsomal incubations.

The concentration of DMDO was not measured in the procedure because DMDO rapidly escaped from the solution, and DMDO measurement is time-consuming (Murray and Singh, 1997). A 1.2-ml-volume of DMDO solution was sufficient to oxidize 0.029 mmol of DB, suggesting that the concentration of DMDO was >0.024 M. UPLC-MS/MS was used, instead of ^1H -NMR as previously reported (Druckova and Marnett, 2006), to monitor the disappearance of DB that was oxidized

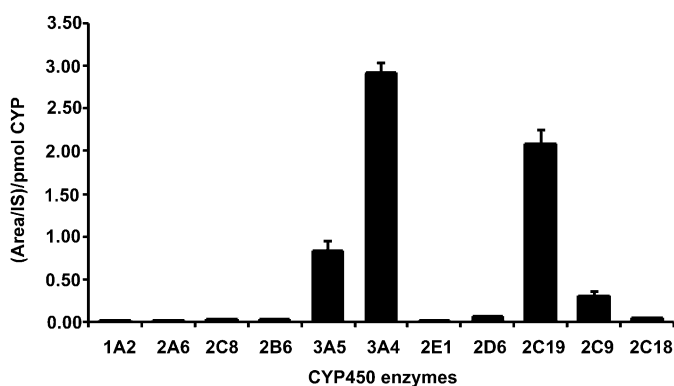


Fig. 11. Formation of metabolite M31 by recombinant human cytochrome P450 isoenzymes after incubation with 50 μ M DB for 60 minutes. Data shown are the area obtained in the mass spectrum for M31 relative to the internal standard and normalized to the amount of P450 (average of triplicate determinations \pm S.D.).

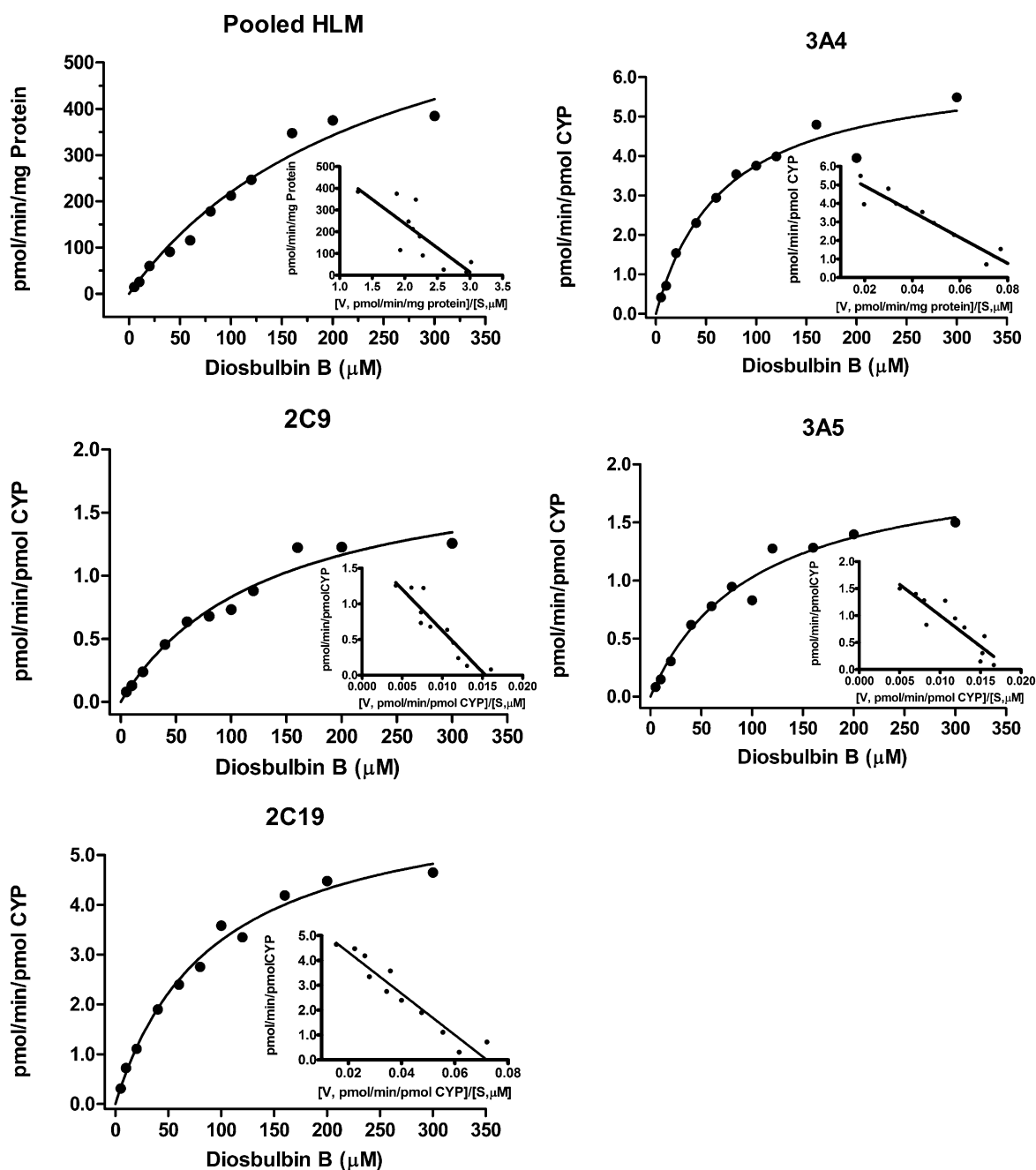


Fig. 12. Enzyme kinetics after incubation with 5–300 μM DB for 60 minutes. Data show the mean of triplicate determinations.

by DMDO. Unexpectedly, several products with signals at m/z 377, including M12 and M13, were detected. Thus, M12 and M13 were successfully synthesized. The reaction was completed in 15 minutes, and the major products (M31a and M31b) transferred to their isomers (M27 and M28) when the reaction continued, which made the separation more difficult. M27 and minor M28 to M30 were hard to separate because they were always eluted together. M35 (m/z 939), having poor stability because of the mercapto group, was hard to obtain as well.

The structures of the reactive metabolites of DB were confirmed by the structure elucidations of metabolites in the trapping experiments of Tris base, methoxylamine, and GSH. Although it was difficult to distinguish metabolites with different configurations, the structure elucidations from mass data and literatures made sense except

that M7 to M9 were temporarily inferred due to their few mass data. Chemical synthesis of M12, M13, M35, and M27 to M31 proved the existence of reactive metabolites, and finally, the NMR data of M12, M13, and M31 confirmed the structures of reactive metabolites.

UPLC-MS/MS with multiple reaction mode was used to improve selectivity and sensitivity to determine MGCD in incubations. MGCD was insoluble in acetonitrile, but soluble in DMSO. DMSO (10%) was used to reconstitute the residual to determine MGCD in enzyme kinetic samples, which resulted in high recoveries (90–110%) of MGCD. Prior to protein precipitation, 10 μl of DMSO was added to keep the same procedure with calibration samples.

CYP3A4 was identified as the major bioactivation enzyme of DB both by chemical inhibition studies and RAF approach. Considering

TABLE 4
Enzyme kinetic parameters for diosbulbin B to M31

HLM and P450s	V_{\max} <i>pmol·min⁻¹·mg⁻¹ protein or pmol·min⁻¹·pmol⁻¹ P450</i>	K_m μM	V_{\max}/K_m <i>l·min⁻¹·mg⁻¹ protein or l·min⁻¹·pmol⁻¹ P450</i>
HLM	776.2	254.0	3.06×10^{-3}
3A4	6.3	68.3	9.22×10^{-5}
3A5	2.0	98.5	2.03×10^{-5}
2C9	1.9	133.8	1.42×10^{-5}
2C19	6.3	92.0	6.85×10^{-5}

that the RAF approach provides a satisfactory prediction of the roles of P450 isoforms compared with inhibition studies, contribution of CYP2C9 to the bioactivation of DB was higher than that of CYP2C19. However, the result in the RAF approach was contrary to those from the inhibition studies. The contrasting results may have been caused by the compensation effects from other enzymes when CYP2C9 was inhibited. The K_m of CYP3A4 in the formation of M31 was 68.1 μM , indicating that DB had moderate affinity to CYP3A4.

DB and teucrin A both are bioactivated in the furan moiety. Furan has already been proved to transform into *cis*-2-butene-1,4-dial by P450 (Chen et al., 1995). This reactive substance, *cis*-2-butene-1,4-dial, is likely responsible for the ultimate toxic and carcinogenic properties of furan because it readily reacts with amino acids (Chen et al., 1997) and DNA (Byrns et al., 2006). Mono-GSH adduct of furan, as well as metabolites derived from cysteine *cis*-2-butene-1,4-dial lysine cross-links, was found in rat urine, which suggested that furan was also bioactivated to form *cis*-2-butene-1,4-dial *in vivo* (Peterson et al., 2006; Lu and Peterson, 2010). A substantial amount of radioactive materials remained in the liver bound to proteins at 24 hours after dosing 8 mg/kg [¹⁴C]furan to rats, which could cause hepatotoxicity in rats (Burka et al., 1991). Hepatotoxicity is correlated with covalent binding to hepatic proteins with reactive metabolites, which also has been clarified from numerous drugs. Acetaminophen is very safe at therapeutic doses. However, acetaminophen overdose may produce fulminating hepatic necrosis that can be fatal. Covalent binding resulting from reactive metabolites is the most important factor (Hinson et al., 2004). Troglitazone, an oral antidiabetic agent, has been removed from the market because it has been associated with cases of severe hepatotoxicity and drug-induced liver failure. Reactive metabolites may play a causative role (Kassahun et al., 2001). The bioactivation of DB that has been inferred to form reactive metabolites by liver P450 might be primarily responsible for the hepatotoxicity of DB and *D. bulbifera*.

Previous studies have demonstrated that extract from *D. bulbifera*, in which DB is the main ingredient, could cause higher hepatotoxicity in male compared with female mice (Wang et al., 2011). Pharmacokinetic studies revealed that the oral absolute bioavailability of DB in female rats was nearly seven times higher than that in males (Yang et al., 2013). Given that DB was mainly metabolized by CYP3A4 in humans, higher expression of 3A, including 3A1 and 3A2, in male than in female mice and rats (Kato and Yamazoe, 1992) could enhance more metabolized DB, resulting in higher hepatotoxicity in male mice and lower oral bioavailability in male rats than in females. Concerning drug interaction, it should be noted that *D. bulbifera* may cause fatal toxicity when used with inducers of CYP3A4 such as rifampicin and hyperforin that also induce CYP2C9 expression (Chen et al., 2004; Harmsen et al., 2009).

The relationship of the reactive metabolites with the antitumor activity of DB remains unknown. P450 expression, particularly 1A, 1B, 2C, 3A, and 2D subfamily members, has been identified in a wide range of human cancers (Patterson and Murray, 2002). Prodrug

antitumor agents that depend on P450 activation may also be activated in tumor regions. For example, AQ4N (1,4-bis-[(2-(dimethylamino-*N*-oxide)ethyl)amino]-5,8-dihydroxy-anthracene-9,10-dione), a CYP3A substrate, is activated to a cytotoxic metabolite specifically in hypoxic tumor regions (Patterson and Murray, 2002). The relationship between reactive metabolites of DB and antitumor activity should be investigated in future studies.

Authorship Contributions

Participated in research design: Yang, Chen, Z. Wang, C. Wang.

Conducted experiments: Yang, Liu.

Contributed new reagents or analytic tools: Yang, Liu, C. Wang.

Performed data analysis: Yang, Liu, C. Wang.

Wrote or contributed to the writing of the manuscript: Yang, Liu, C. Wang.

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