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Mulberrofuran G and Isomulberrofuran G from *Morus alba* L.: Anti-hepatitis B Virus Activity and Mass Spectrometric Fragmentation

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Supporting Information

ABSTRACT: Mulberrofuran G (**1**) and isomulberrofuran G (**2**), a pair of isomeric Diels–Alder-type adducts, were isolated from the root bark of *Morus alba* L. Isomulberrofuran G (**2**) as a new IIB-type Diels–Alder-type adduct, was elucidated by extensive ^{1}H, ^{13}C, and two-dimensional (2D) nuclear magnetic resonance (NMR) and mass spectrometry (MS) spectroscopic analyses. A fragmentation study on compounds **1** and **2** was performed by high-resolution electrospray ionization (ESI) multistage tandem mass spectrometry linked with ion-trap (IT) and time-of-flight (TOF) mass analyzers (ESI–MS^n/IT–TOF) in negative mode, which resulted in obviously different fragmentations. In the MS^2 experiments, the characteristic ions at m/z 451 and 439 could be revealed as their respective diagnostic ions. Mulberrofuran G (**1**) showed moderate activity, inhibiting hepatitis B virus (HBV) DNA replication with the IC_{50} value of 3.99 μM, according to the anti-HBV assay on the HepG 2.2.15 cell line *in vitro*.

KEYWORDS: *Morus alba, isomulberrofuran G, Diels–Alder-type adducts, MS^n fragmentation study, LCMS–IT–TOF, anti-HBV activity*

INTRODUCTION

Morus alba L. is a useful plant widely cultivated in China for thousands of years, whose leaves are indispensable food for silkworms and fruits (Sang-Shen in Chinese) are largely consumed in normal diets.[1] Its root bark (Mori Cortex), leaves, and twigs are all well-known traditional Chinese medicines (TCMs) documented in *Chinese Pharmacopoeia*.[2] In particular, Mori Cortex is famous with the Chinese name of "Sang-Bai-Pi" and widely used for anti-inflammation, anti-hypertension, hypoglycemic, and diuretic purposes.[3] The earliest usage of Mori Cortex can be traced back to "Shen-Nong-Ben-Cao-Jing", the first Chinese dispensatory, which probably appeared at the end of the third century.[4] Mori Cortex is rich in phenolic compounds, especially for isoprenylated flavonoids and Diels–Alder-type adducts, which have aroused much interest of scientists for their complicated structures and diverse activities.[5−8] Up to now, more than 50 Diels–Alder-type adducts with hypotensive, hypoglycemic, antioxidative, and anti-human immunodeficiency virus (HIV) activities have been isolated from Moraceous plants.[9−14] Diels–Alder-type adducts derived from [4 + 2]cyclo-adducts of chalcones as dienophiles and dehydroprenylphenols as dienes are the first examples of natural products biosynthesized by the enzyme-controlled intermolecular Diels–Alder reaction.[15] The regioselectivity of the Diels–Alder reaction, including *exo* and *endo* addition, results in all-*trans* and *cis*–*trans* types of Diels–Alder-type adducts. Structurally, Diels–Alder-type adducts can be classified into two types based on the phenol nuclei: (I) adducts of one chalcone and one dehydroprenylflavone and (II) adducts of one chalcone and one dehydroprenylstilbene. In the case of type II, *para*- or *meta*- dehydroprenylstilbenes will produce two subgroups IIA and IIB. The keto carbonyl group in the chalcone part can be further cyclized with the intramolecular hydroxy groups by the ketol reaction.[16,17] To search for more Diels–Alder-type adducts from Mori Cortex and characterize their bioactivities and mass spectrometry (MS) fragmentation regularities, this investigation was designed to isolate and determine the structure and multistage tandem mass spectrometry (MS^n) fragmentation of Diels–Alde-type adducts from *M. alba* and study their anti-hepatitis B virus (HBV) properties.

MATERIALS AND METHODS

General Experimental Procedures. One-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectra were recorded on Bruker DRX-500 and AVANCE III-600 spectrometers (Bruker, Bremerhaven, Germany) with tetramethylsilane (TMS) as the internal standard. MS data were collected on Shimadzu liquid chromatography–mass spectrometry (LCMS)–ion-trap (IT)–time of flight (TOF) (Shimadzu, Kyoto, Japan). Infrared (IR) (KBr) spectra were recorded on a Bio-Rad FTS-135 spectrometer (Bio-Rad, Hercules, CA). Ultraviolet (UV) data were collected on a Shimadzu UV-2401A spectrophotometer (Shimadzu, Kyoto, Japan). Optical rotations were collected on a Jasco model 1020 polarimeter (Horiba, Tokyo, Japan). Silica gel (Makall, Qingdao, China) and Toyopearl HW-40C gel (Toson, Tokyo, Japan) were used for column chromatography.

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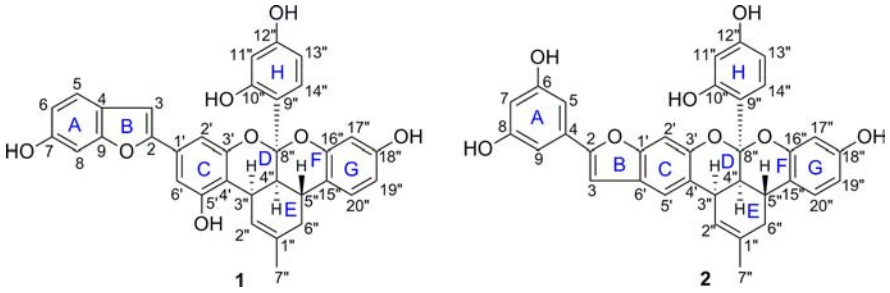

Figure 1. Structures of mulberrofuran G (**1**) and isomulberrofuran G (**2**).

Table 1. ^{1}H and ^{13}C NMR Data of Isomulberrofuran G (2)[a]

number	δ_H	δ_C	number	δ_H	δ_C
2		156.4, s	**6″a**	2.05, 1H, dd, 16.5, 11.0	36.4, t
			6″b	2.71, 1H, dd, 16.5, 5.5	
3	7.03, 1H, s	102.1, d	**7″**	1.80, 3H, brs	23.8, q
4		133.2, s	**8″**		102.4, s
5	6.86, 1H, d, 2.0	103.9, d	**9″**		117.5, s
6		159.9, s	**10″**		157.4, s
7	6.37, 1H, t, 2.0	103.8, d	**11″**	6.43, 1H, d, 2.4	104.7, d
8		159.9, s	**12″**		160.0, s
9	6.86, 1H, d, 2.0	103.9, d	**13″**	6.21, 1H, dd, 8.8, 2.4	107.1, d
1′		155.1, s	**14″**	7.26, 1H, d, 8.8	130.5, s
2′	7.11, 1H, s	99.3, d	**15″**		117.3, s
3′		150.8, s	**16″**		153.5, s
4′		122.4, s	**17″**	6.37, 1H, d, 2.5	104.1, d
5′	7.41, 1H, s	121.1, d	**18″**		157.8, s
6′		124.6, s	**19″**	6.48, 1H, dd, 8.3, 2.5	109.9, d
1″		135.0, s	**20″**	7.09, 1H, d, 8.3	127.9, d
2″	6.12, 1H, brd, 5.0	123.5, d	**OH-6/OH-8**	8.54, 2H, s	
3″	3.44, 1H, dd, 5.5, 5.0	35.3, d	**OH-10″**	8.64, 1H, s	
4″	3.36, 1H, dd, 11.5, 5.5	36.5, d	**OH-12″**	8.51, 1H, s	
5″	2.88, 1H, ddd, 11.5, 11.5, 5.5	27.8, d	**OH-18″**	8.40, 1H, s	

[a]Data were measured in acetone-d_6, with δ in ppm and J in Hz.

Electrospray ionization (ESI)−MS^n spectra were acquired on a Shimadzu LCMS−IT−TOF mass spectrometer equipped with an ESI source in negative mode. The mass resolution was about 10 000 full width at half maximum (fwhm). Accurate masses were corrected by calibration using the sodium trifloroacetate (CF_3CO_2Na) clusters. Unless specified otherwise, analytical conditions were as follows: spray voltage, −3.50 kV; detector voltage, 1.70 kV; pressure of the TOF region, 1.6×10^{-4} Pa; pressure of the IT, 1.5×10^{-2} Pa; rotary pump (RP) area vacuum, 65.5 Pa; dry gas pressure, 100.0 kPa; nebulizing gas (N_2) flow, 0.5 L/min; curved desolvation line (CDL) temperature, 200 °C; heat block temperature, 200 °C; equipment temperature, 40 °C; precursor-ion-selected width, m/z 3.0, selected time, 20 ms; collision-induced dissociation (CID) collision time, 20 ms; collision energy, 50%; collision gas, 50%; and q, 0.251. The Shimadzu composition formula predictor was used to speculate the molecular formula. Sample solutions of mulberrofuran G (**1**) and isomulberrofuran G (**2**) were prepared by dissolving each sample in a solution of acetonitrile (0.5% Et_2NH) to a final concentration of 0.1 mg/mL. The sample was introduced into the source via a syringe pump at a flow rate of 1 μL/min.

Plant Material. The root bark of *M. alba* L. (Sang-Bai-Pi) was purchased from Jü-Hua-Cun Medicinal Herb Market, Kunming, Yunnan, China, and was identified by Prof. Li-Gong Lei (Kunming Institute of Botany, Chinese Academy of Sciences). A voucher specimen (2011-01-02) was deposited in the Laboratory of Antivirus and Natural Medicine Chemistry, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. The powdered root bark of *M. alba* (2.0 kg) was soaked in EtOH (95%) and extracted under reflux 2 times, at 2 h for each time. The combined EtOH extracts (150.0 g) were condensed and partitioned between H_2O and EtOAc. The EtOAc part was separated by silica gel column chromatography (2.0 kg, 50.0 × 11.0 cm inner diameter, 200−300 mesh), eluted with the $CHCl_3$−MeOH system (each 20 L, from 100:0 to 50:50, v/v). The $CHCl_3$−MeOH (90:10) eluted portion (10.0 g) was further subjected on silica gel column chromatography (100.0 g, 50.0 × 2.5 cm inner diameter, 200−300 mesh) using a $CHCl_3$−Me_2CO gradient (each 1.0 L), from which the $CHCl_3$−Me_2CO (80:20) fraction was obtained. This fraction was purified by repeated column chromatography (silica gel and Toyopearl HW-40C gel) to yield mulberrofuran G (**1**, 100 mg) and isomulberrofuran G (**2**, 20 mg), with the purity higher than 95% (Figure 1).

Isomulberrofuran G (2). Brownish yellow powder. $[\alpha]^{24}_D$ +514.8 (*c* 0.2, MeOH). UV (MeOH) λ_{max} (log ε): 335 (4.46), 322 (4.52), 296 (4.16), 284 (4.21) nm. IR (KBr) ν_{max}: 3375, 2970, 1623, 1510, 1455, 1353, 1284, 1258, 1104, 1020, 967, 841 cm^{-1}. ^{1}H and ^{13}C NMR data (Table 1). HRESIMS m/z: 561.1570 $[M - H]^-$ (calcd for $C_{34}H_{25}O_8$, 561.1555).

Anti-HBV Assays. The procedure for the anti-HBV screen was performed in accordance with our previous reports.[18] Anti-HBV activity and cytotoxicity of mulberrofuran G (**1**) and isomulberrofuran G (**2**) were assayed on the HepG 2.2.15 cell line *in vitro*, which was stably transfected with the HBV genome using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The activity inhibiting HBV antigen [hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg)] secretion and HBV DNA replication was analyzed by enzyme-linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (PCR) methods, and cytotoxicity was measured by a

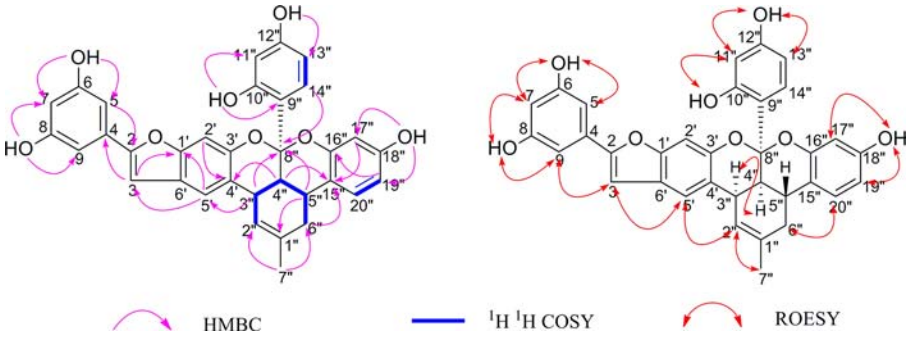

Figure 2. Selected 2D NMR correlations for isomulberrofuran G (**2**).

Table 2. Accurate Masses and Elemental Compositions of Mulberrofuran G (1) and Isomulberrofuran G (2) from Negative ESI−IT−TOF MS^n Experiments

MS^n	precursor ion	product ion	calculated m/z	measured m/z	error m/z	error ppm	elemental composition
MS	mulberrofuran G (**1**)	561	561.1555	561.1536	−1.9	−3.39	$C_{34}H_{25}O_8$
MS^2	561 ([M − H]$^-$)	451	451.1187	451.1153	−3.4	−7.54	$C_{28}H_{19}O_6$
MS^3	561−451	436	436.0952	436.0949	−0.3	−0.69	$C_{27}H_{16}O_6$
MS^3	561−451	437	437.1031	437.0991	−4.0	−9.15	$C_{27}H_{17}O_6$
MS^4	561−451−437	408	408.1003	408.1006	0.3	0.74	$C_{26}H_{16}O_5$
MS^5	561−451−437−408	381	381.1132	381.1119	−1.3	−3.41	$C_{25}H_{17}O_4$
MS^6	561−451−437−408−381	352	352.1105	352.1144	3.9	11.08	$C_{24}H_{16}O_3$
MS^7	561−451−437−408−381−352	324	324.1156	324.1124	−3.2	−9.87	$C_{23}H_{16}O_2$
MS	isomulberrofuran G (**2**)	561	561.1555	561.1570	1.5	2.67	$C_{34}H_{25}O_8$
MS^2	561 ([M − H]$^-$)	439	439.1187	439.1160	−2.7	−6.15	$C_{27}H_{19}O_6$
MS^3	561−439	422	422.1160	422.1136	−2.4	−5.69	$C_{27}H_{18}O_5$
MS^4	561−439−422	379	379.0976	379.0965	−1.1	−2.90	$C_{25}H_{15}O_4$
MS^5	561−439−422−379	337	337.0870	337.0885	1.5	4.45	$C_{23}H_{13}O_3$
MS^3	561−439	396	396.1367	396.1338	−2.9	−7.32	$C_{26}H_{20}O_4$
MS^4	561−439−396	355	355.0976	355.0980	0.4	1.13	$C_{23}H_{15}O_4$
MS^4	561−439−396	311	311.1078	311.1057	−2.1	−6.75	$C_{22}H_{15}O_2$
MS^5	561−439−396−355	311	311.1078	311.1037	−4.1	−13.18	$C_{22}H_{15}O_2$
MS^5	561−439−396−311	282	282.1050	282.1026	−2.4	−8.51	$C_{21}H_{14}O$
MS^6	561−439−396−311−282	254	254.0737	254.0689	−4.8	−18.89	$C_{19}H_{10}O$

modified (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Samples were dissolved in dimethylsulfoxide (DMSO) for anti-HBV activity and cytotoxicity assays, with the concentration below 2.5 μL/mL not affecting the growth of cells.

RESULTS AND DISCUSSION

Structural Identification. Isomulberrofuran G (**2**) was isolated as a brownish yellow powder, with the molecular formula of $C_{34}H_{26}O_8$ identical to mulberrofuran G (**1**), which was determined by negative HRESIMS ([M − H]$^-$). The ^{13}C [distortionless enhancement by polarization transfer (DEPT)] spectrum displayed 34 carbons, involving 1 methyl, 1 methylene, 16 methines, and 16 quaternary carbons. In ^{1}H NMR spectrum, five hydroxy groups were recognized at the downfield and confirmed by the heteronuclear singular-quantum correlation (HSQC) experiment. Two hydroxy groups overlapped at δ_H 8.54 (OH-6 and OH-8) in combination with the aromatic protons at δ_H 6.86 (2H, d, J = 2.0 Hz, H-5, H-9) and 6.37 (1H, d, J = 2.0 Hz, H-7), indicating a symmetric 3,5-dihydroxyphenyl moiety. Two characteristic ABX spin systems were denoted by the resonances at δ_H 6.43 (1H, d, J = 2.4 Hz, H-11″), 6.21 (1H, dd, J = 8.8, 2.4 Hz, H-13″), and 7.26 (1H, d, J = 8.8 Hz, H-14″) and at δ_H 6.37 (1H, d, J = 2.5 Hz, H-17″), 6.48 (1H, dd, J = 8.3, 2.5 Hz, H-19″), and 7.09 (1H, d, J = 8.3 Hz, H-20″), which were determined as two 2,4-dihydroxyphenyl moieties. Three singlet peaks at δ_H 7.03 (1H, s, H-3), 7.11 (1H, s, H-2′), and 7.41 (1H, s, H-5′) were displayed in the ^{1}H NMR spectrum, indicating three isolated aromatic protons. Isomulberrofuran G (**2**) was proposed to have the same ring *E* portion with mulberrofuran G (**1**) from ^{1}H− ^{1}H (homonuclear chemical shift) correlation spectroscopy (COSY) (H-2″/H-3″/H-4″/H-5″/H-6″), heteronuclear multiple-bond correlation (HMBC) (H-7″/C-2″ and C-6″ and H-3″ and H-5″/C-1″), and rotating frame Overhauser effect spectroscopy (ROESY) (H-4″/H-3″, H-4″/6″α, and H-5″/H-6″β) experiments, as well as the almost identical NMR data and coupling constants (J) of H-3″, H-4″, H-5″, and H-6″ as those of mulberrofuran G (**1**). Similarly, the identical rings D, F, G, and H were constructed on the basis of extensive 1D and 2D NMR spectrometric analyses (Table 1 and Figure 2). A 2-(3,5-dihydroxyphenyl)benzofuran moiety (rings A−C) was completed by HMBC correlations of H-5 with C-2, H-3 with C-4 and C-1′, H-5′ with C-3 and C-1′, and H-2′ with C-4′ and C-6′, together with ROESY cross-peaks of OH-6/H-5 and H-7, OH-8/H-7 and H-9, H-9/H-3, and H-3/H-5′. The detected ROESY correlation of H-5′ with H-2″ and HMBC correlations of H-2′ with C-4′ and C-6′ were important for the determination of the linear coupled mode for rings B, C, and D. The above analyses were further confirmed by comparing its NMR data to those of australisin C,[19] indicating

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Table 3. Anti-HBV Activity and Cytotoxicity of Mulberrofuran G (1) and Isomulberrofuran G (2)[a]

	MTT	HBsAg		HBeAg		HBV DNA	
compounds	CC_{50}	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI
mulberrofuran G (1)	8.04 ± 1.00	14.34 ± 0.31	<1	61.55 ± 3.57	<1	3.99 ± 2.28	2.0
isomulberrofuran G (2)	6.79 ± 0.24	33.77 ± 4.59	<1	183.19 ± 26.11	<1	9.10 ± 2.37	<1
tenofovir[b]	>1740.89	>1740.89	c	>1740.89	c	2.54 ± 0.14	>685.4

[a]Results are expressed as the mean (μM) ± standard deivation (SD) from three independent experiments. [b]Tenofovir was used as the positive control. [c]CC_{50} and IC_{50} were not reached at the highest tested concentration.

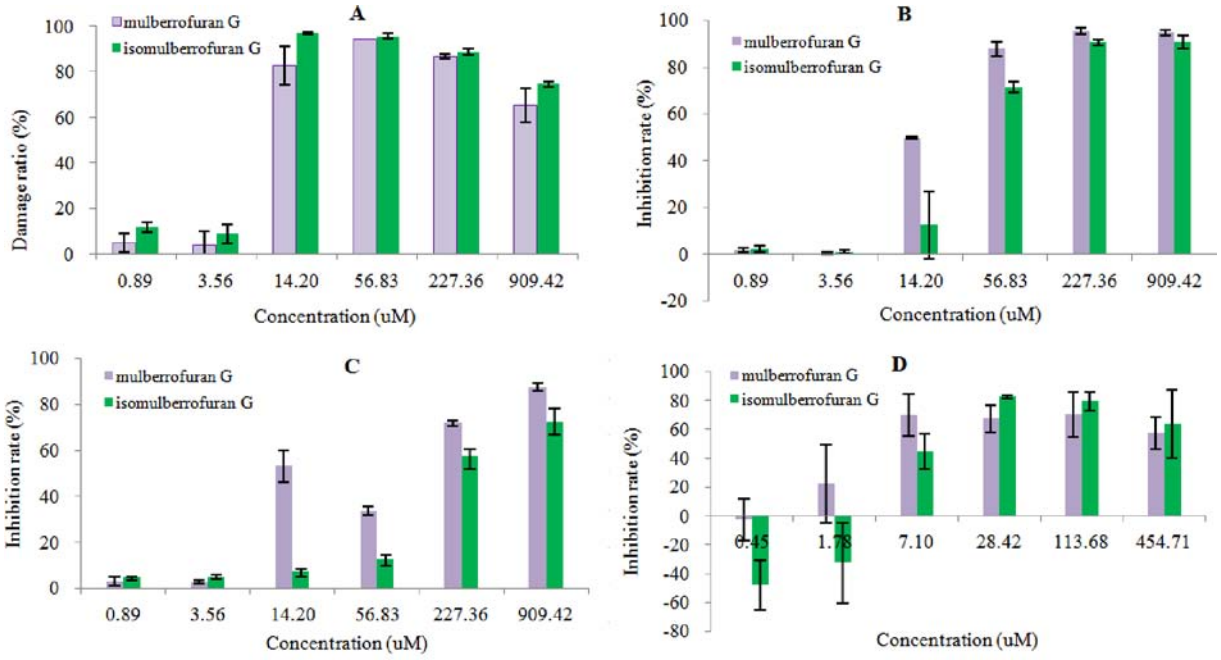

Figure 3. Cytotoxicity and anti-HBV activity of mulberrofuran G (1) and isomulberrofuran G (2) on HepG 2.2.15 cells: (A) cytotoxicity, (B) anti-HBsAg activity, (C) anti-HBeAg activity, and (D) anti-HBV DNA activity. Results are expressed as the mean ± SD from three independent experiments. Significant difference: $p < 0.05$.

the same rings A−C. Thus, the structure of isomulberrofuran G (2) was constructed and confirmed by the HMBC, $^1H-^1H$ COSY, and ROESY analyses (Figure 2). It should be noted that the correlations between the hydroxy groups and adjacent aromatic protons in the ROESY experiment played important roles in structural determination, which benefited from the hydroxy signals that appeared in the ^1H NMR experiment (acetone-d_6).

MS^n Fragmentation Study. Mulberrofuran G (1) and isomulberrofuran G (2) are a pair of isomers with identical molecular formula of $C_{34}H_{26}O_8$ and similar spectroscopic properties. Structurally, they occupy a complicated system consisting of eight rings with five hydroxy groups, and the main difference is located at rings A−C. The similar structural features and spectroscopic properties present challenges for rapid and accurate differentiation of them. A mass spectrometer equipped with an ESI source and IT analyzer coupled to a TOF mass analyzer (ESI−IT−TOF) is effective for the fragmentation study on complicated compounds, which enables multistage tandem spectra (MS^{1-10}) with high accuracy and resolution in both MS and MS^n modes.[20,21] Up to date, no extensive MS fragmentation study has been conducted on Diels−Alder-type adducts. Therefore, the first-time characterization of the fragmentation patterns of mulberrofuran G (1) and isomulberrofuran G (2) will provide valuable information for identification and differentiation of Diels−Alder-type adducts.

The accurate mass spectrometric data of compounds **1** and **2** were obtained from 16 multistage tandem MS experiments in negative ESI mode. Generally, the most abundant ion (base peak) or the product ion with the highest mass (excluding adducts with a larger mass than that of the precursor ion) in each spectrum was subjected to the next stage analysis. The accurate masses and corresponding assigned elemental compositions of the product ions from the tandem MS analyses were summarized in Table 2. The accuracy of obtained masses relative to the assigned structures was always within ±5 mDa or 10 ppm error, which made the calculated elemental compositions reliable.

In the single-stage mass experiments, using full-scan acquisition (m/z 100−1000) in negative-ion mode, both the deprotonated molecular ions $[M - H]^-$ and adduct ions $[M + Cl]^-$ were readily observed for mulberrofuran G (1) and isomulberrofuran G (2). When their respective $[M - H]^-$ ion was selected as the precursor ion to perform MS^2 analysis, different product ions at m/z 451.1153 for compound **1** and m/z 439.1160 for compound **2** were detected. This distinction resulted in obviously different fragmentations in the subsequent multistage tandem MS^n analyses (Table 2). Therefore, the product ions at m/z 451.1153 and 439.1160 in MS^2 spectra could be recognized as the diagnostic ions for mulberrofuran G

(**1**) and isomulberrofuran G (**2**), from which they can be rapidly and accurately distinguished.

Anti-HBV Investigation. To evaluate the anti-HBV properties of the isolates, mulberrofuran G (**1**) and isomulberrofuran G (**2**) were assayed on the HepG 2.2.15 cell line *in vitro*. From the data presented in Table 3 and Figure 3, compound **1** exhibited moderate activity against HBV DNA replication, with the IC_{50} value of 3.99 μM [selective index (SI) = 2.0]. Although mulberrofuran G (**1**) and isomulberrofuran G (**2**) showed potent anti-HBV DNA activities with low IC_{50} values equivalent to that of tenofovir, their obvious cytotoxicity (CC_{50} value is 8.04 and 6.79 μM, respectively) resulted in low selectivity. At low concentrations (<3.56 μM), compounds **1** and **2** exhibited weak cytotoxicity; however, the damage ratio to HepG 2.2.15 cells increased rapidly with the concentration up to 14.20 μM. Since then, the cytotoxicity was not increased with the concentration increasing (from 14.20 to 909.42 μM). The anti-HBV activities of compounds **1** and **2** showed similar trends to their cytotoxicity. Thus, it will be very interesting to perform an extensive structure−modification study on mulberrofuran G (**1**) and isomulberrofuran G (**2**) to decrease their cytotoxicity for developing novel anti-HBV agents.

Briefly, mulberrofuran G (**1**), belonging to the IIA-type Diels−Alder-type adduct, was initially isolated from *Morus lhou*,[22] whereas its isomer isomulberrofuran G (**2**) is the first example of the IIB-type Diels−Alder-type adduct with ketolization. The tandem mass spectrometric study on mulberrofuran G (**1**) and isomulberrofuran G (**2**) resulted in obviously different MS fragmentation regularities and their respective diagnostic ions at *m*/*z* 451.1153 and 439.1160. Mulberrofuran G (**1**) showed potent activity, inhibiting HBV DNA replication with the IC_{50} value of 3.99 μM based on the anti-HBV assay on the HepG 2.2.15 cell line *in vitro*. To our knowledge, this is the first report on the anti-HBV constituents of Mori Cortex and MS^n fragmentation rules of Diels−Alder-type adducts, which will provide valuable information for the further understanding of *M. alba*.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H, ^{13}C (DEPT), and 2D NMR (HSQC, HMBC, $^1H-^1H$ COSY, and ROESY), HRESIMS, IR, UV, and $[\alpha]_D$ spectra for isomulberrofuran G (**2**), as well as the MS^n spectra for mulberrofuran G (**1**) and isomulberrofuran G (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CID, collision-induced dissociation; COSY, (homonuclear chemical shift) correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer; ESI, electrospray ionization; fwhm, full width at half maximum; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear singular-quantum correlation; ROESY, rotating frame Overhauser effect spectroscopy; SI, selective index; TOF, time of flight

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