First Example of Diterpenoids from 14,15-Cyclopimarane in the Roots of *Linum usitatissimum*

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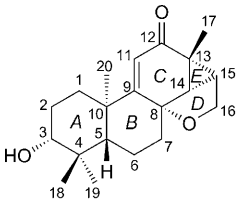
Chemical investigation of the roots of *Linum usitatissimum* led to the isolation and characterization of a new diterpenoid, named usitatissimin A (**1**), together with four known compounds. The structure of the new compound was established by spectroscopic methods, including X-ray crystallographic diffraction analysis that confirmed the relative configuration of **1**. This is the first example of a diterpenoid with a novel 6/6/6/3-membered ring system of 14,15-cyclopimarane. In addition, usitatissimin A showed pronounced hepatoprotective activity against D-galactosamine-induced toxicity in WB-F 344 rat hepatic epithelial stem-like cells.

Introduction. – Diterpenoids are terpenoid compounds that represent a large, diverse, and unique class of non-volatile C_{20} products. The advances in the discoveries of new diterpenoids have regularly been reviewed by *Hanson*, and the vast majority of diterpenoids are acyclic and carbocyclic compounds containing as many as five rings [1–4]. We report herein a novel tetracarbocyclic diterpenoid, isolated in a pure state from the roots of *Linum usitatissimum* L. (Linaceae) collected in the northeast of China, when we aimed to discover new active hepatoprotective compounds in this plant. The new compound includes an additional saturated tetrasubstituted furan ring and possesses a new kind of C-skeleton and was named as usitatissimin A (**1**). This 14,15-cyclopimarane skeleton is here given the trivial name usitalinpimarane which is based on plant origin and C-skeleton. In addition, pyrrolidine-2,5-dione 5-oxime (**2**), together with three other known compounds, genkwanin-4′-*O*-*β*-D-glucopyranoside, maleimide-5-oxime (= 5-(hydroxyimino)-1,5-dihydro-2*H*-pyrrol-2-one), and glaberide I, were also isolated and identified. D-Galactosamine-induced toxicity in WB-F 344 was evaluated for compounds **1**, **2**, and genkwanin-4′-*O*-*β*-D-glucopyranoside.



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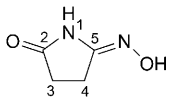

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Results and Discussion. – The structure of usitatissimin A was assigned as 3α-hydroxy-8α,16-epoxy-14,15-cyclopimar-9(11)-en-12-one on the basis of the following findings. It was isolated as a crystalline solid. The HR-EI-MS spectrum of usitatissimin A exhibited the molecular ion peak at m/z 316.2032 (M^+), corresponding to the molecular formula $C_{20}H_{28}O_3$. The IR spectrum (KBr) showed OH, hydrate, and α,β-unsaturated ketone bands, respectively. The ^{13}C-NMR and DEPT spectra (*Table 1*) revealed that the basic C-atom skeleton consisted of four Me, five CH_2, and five CH

Table 1. *^{1}H-* (500 MHz) *and ^{13}C-NMR* (125 MHz) *Data of Usitatissimin A* (**1**)

	δ(H)	δ(C) (DEPT)	^{1}H,^{1}H-COSY Correlations	HMBC
H_α–C(1)	1.89 (*dt*, *J* = 13.0, 3.5)	35. 8 (*t*)	H_β–C(1), H_α–C(2), H_β–C(2)	C(2), C(3), C(5), C(10)
H_β–C(1)	1.43 (*td*, *J* = 13.0, 3.5)		H_α–C(1), H_α–C(2), H_β–C(2)	C(20)
H_α–C(2)	1.75–1.79 (*m*)	27.3 (*t*)	H_α–C(1), H_β–C(1), H_β–C(2), H–C(3)	C(3), C(10)
H_β–C(2)	*ca.* 1.70 overlapped		H_α–C(1), H_β–C(1), H_α–C(2), H–C(3)	C(3), C(4), C(10)
H–C(3)	3.21 (*dd*, *J* = 11.5, 4.5)	78.1 (*d*)	H_α–C(2), H_β–C(2)	C(18), C(19)
C(4)		39.4 (*s*)		
H–C(5)	1.11 (*dd*, *J* = 8.5, 6.0)	51.1 (*d*)	CH_2(6)	C(1), C(4), C(7), C(10)
CH_2(6)	*ca.* 1.70 overlapped	18.4 (*t*)	H–C(5), H_α–C(7), H_β–C(7)	C(4), C(5), C(7), C(8), C(10)
H_α–C(7)	2.35 (*dt*, *J* = 14.0, 2.5)	38.7 (*t*)	CH_2(6), H_β–C(7)	C(5), C(8), C(9), C(14)
H_β–C(7)	1.92–1.98 (*m*)		CH_2(6), H_α–C(7)	C(5), C(6), C(8), C(14)
C(8)		79.2 (*s*)		
C(9)		164.1 (*s*)		
C(10)		40.0 (*s*)		
H–C(11)	5.99 (*s*)	123.1 (*d*)		C(8), C(9), C(10), C(13)
C(12)		198.2 (*s*)		
C(13)		36.2 (*s*)		
H–C(14)	*ca.* 2.19 overlapped	46.0 (*s*)	overlapped with H–C(15)	C(7), C(8), C(9), C(15), C(17)
H–C(15)	*ca.* 2.19 overlapped	40.9 (*s*)	H_α–C(16), H_β–C(16)	C(8), C(17)
H_α–C(16)	3.40 (*dd*, *J* = 11.0, 4.0)	65.6 (*t*)	H–C(15), H_β–C(16)	C(13), C(15)
H_β–C(16)	4.05 (*dd*, *J* = 11.0, 5.5)		H–C(15), H_α–C(16)	C(8), C(13), C(14), C(15)
Me(17)	1.18 (*s*)	19.5 (*q*)		C(12), C(13), C(14), C(15)
Me(18)	1.03 (*s*)	28.2 (*q*)		C(3), C(4), C(5), C(19)
Me(19)	0.83 (*s*)	15.6 (*q*)		C(3), C(4), C(5), C(18)
Me(20)	1.14 (*s*)	23.2 (*q*)		C(1), C(5), C(9), C(10)

groups, as well as six quaternary C-atoms. Among the 20 C-atoms, there were three sp^2-hybridized C-atoms, including one ketone CO group, which resonated at δ(C) 198.2 (*s*) and one C=C bond at δ(C) 123.1 (*d*) and 164.1 (*s*). In addition, three O-bearing C-atoms were observed at δ(C) 79.2 (*s*), 78.1 (*d*), and 65.6 (*t*). From the above mentioned C-atoms, three are H-bearing and were further confirmed by the resonances at δ(H) 5.99 (*s*, 1 H), 4.05 (*dd*, $J = 11.0, 5.5$, 1 H), and 3.40 (*dd*, $J = 11.0, 4.0$, 1 H), and 3.21 (*dd*, $J = 11.5, 4.5$, 1 H) in the ^{1}H-NMR spectrum (*Table 1*), as well as by the cross-peaks between δ(H) 5.99 and δ(C) 123.1, δ(H) 4.05 and 3.40 and δ(C) 65.6, and δ(H) 3.21 and δ(C) 78.1 in the HSQC experiment. To accommodate seven degrees of unsaturation implied by the molecular formula, usitatissimin A was proposed to be a pentacyclic diterpenoid, and one of the three O-functions within it was determined to be in the form of an ether. In the ^{1}H-NMR spectrum, the four high field Me groups (δ(H) 0.83 (*s*), 1.03 (*s*), 1.14 (*s*), and 1.18 (*s*)) were readily recognized. Except for the four Me groups, signals for three sp^2-hybridized C-atoms and four other quaternary C-atoms were observed. In addition, the three partial structures shown in *Fig. 1* could be identified. With the aid of the DEPT NMR, as well as the detailed ^{1}H,^{1}H-COSY, HSQC, and HMBC experiments, all of the ^{1}H- and ^{13}C-NMR signals could be assigned. Interpretation of the ^2J and ^3J couplings in the HMBC experiment established the connections of these partial structures, which contained mainly the cross-peaks between signals of Me(17) and those of C(12), C(13), C(14), and C(15), of Me(20) and those of C(1), C(5), C(9), and C(10), of Me(18) and those of C(3), C(4), C(5), and C(19), of Me(19) and those of C(3), C(4), C(5), and C(18), of H–C(11) and those of C(8), C(9), C(10), and C(13), of H_α–C(16) and those of C(13) and C(15), of H_β–C(16) and those of C(8), C(13), C(14), and C(15), and of H–C(3) and those of C(18) and C(19). These data not only enabled the OH group to be located at C(3), the C=O group at C(12), the closure of the ether ring at C(8) and C(16), and the presence of geminal dimethyl groups, but led to a tetracarbocyclic diterpene skeleton for usitatissimin A with a novel 6/6/6/3-membered ring system, as well as an extra 2,2,3,4-tetrasubstituted tetrahydrofuran ring. The relatively downfield ^{1}H- and ^{13}C-NMR chemical shifts of C(13), H–C(14), and H–C(15) confirmed the direct connection of the cyclopropane with the α,β-unsaturated ketone group [5][6]. Therefore, the constitutional formula of usitatissimin A was identified.

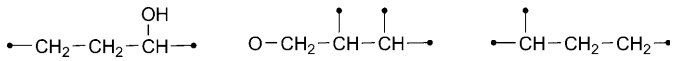


Fig. 1. *Partial structures of usitatissimin A.* •: quaternary C-atoms.

The relative configuration of usitatissimin A was established by NOE difference spectra and with the size of the coupling constants in the ^{1}H-NMR spectrum. The equatorial position of the OH group at C(3) was deduced by the observation of the coupling constants of the *dd* at δ(H) 3.21 ($J = 11.5, 4.5$, H_β–C(3)), and the equatorial positions of H_α–C(1) and H_α–C(7) were also deduced form their ^{1}H-NMR-signal splittings at δ(H) 1.89 (*dt*, $J = 13.0, 3.5$, H_α–C(1)) and 2.35 (*dt*, $J = 14.0, 2.5$, H_α–C(7)). Starting from a conventional α-orientation of Me(20) in pimarane, NOE correlations of

Me(20) with Me(19), H_α–C(2), and H_α–C(16), and of H–C(3) with H_β–C(1), H–C(5), H_β–C(2), and Me(18) confirmed the *trans*-ring fusion of ring *A* and *B*, and the α-orientation of Me(19) and HO–C(3), and the 2,2,3,4-tetrasubstituted tetrahydrofuran ring as well as of the cyclopropane ring (*Fig. 2*). The structure and relative configuration of usitatissimin A were confirmed by a single-crystal X-ray diffraction analysis (*Fig. 3*). The X-ray crystallography affirmed the relative configuration of usitatissimin A, and the result was consistent with the data from the NOE difference spectra. In addition, it was also confirmed that ring *A* and *B* are in a chair conformation, ring *C ca.* in a half-chair conformation, and ring *D* in the envelop conformation. *A*/*B* Rings were found to be fused *trans* and the *C*/*D* and *C*/*E* rings fused *cis*. On the other hand, no intramolecular H-bond was found, but the intermolecular H-bonds between usitatissimin A and a H_2O molecule were observed: $O_2 \cdots O_{1W}$ ($-x+1/2,\ -y+1,\ z+1/2$) 2.705 Å, $O_{1W} \cdots O_1$ 2.854 Å, $O_{1W} \cdots O_2$ ($-x+1,\ y-1/2,\ -z+1/2$) 2.735 Å (*Fig. 4*).

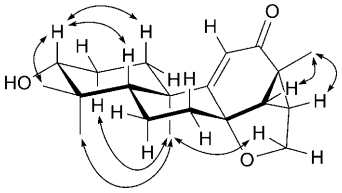


Fig. 2. *Key NOE correlations of usitatissimin A*

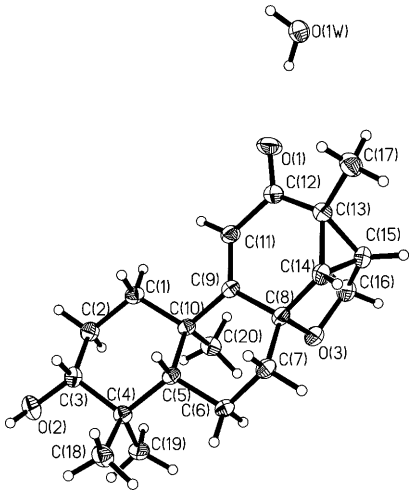


Fig. 3. *Crystal structure of usitatissimin A* (biogenetic numbering)

The structural elucidation of usitatissimin A further exhibited the biodiversity of biogenesis of polycyclic diterpenoids, and the possible biosynthesis of this 6/6/6/3-membered ring system should be considered to be one of the multiple cyclizations and rearrangements from the bicyclic labdane precursor *ent*-copalyl diphosphate (**3**) beginning with an S_N' ring closure that forms the ring *C* (see the *Scheme*) [7]. The co-

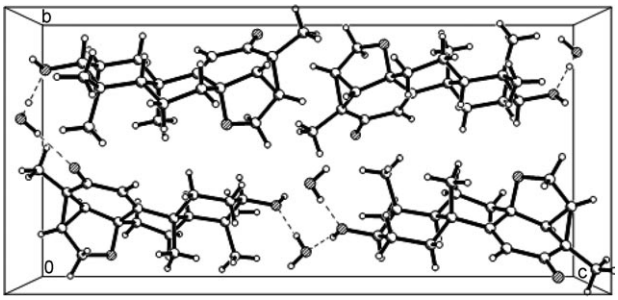
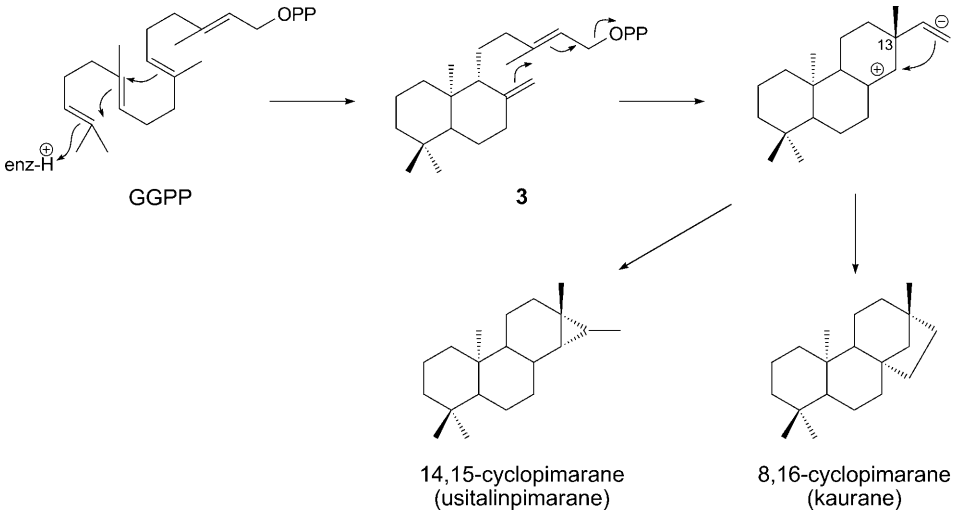


Fig. 4. *View along the* a-*direction of the unit cell*

Scheme. *Proposed Mechanism for the Formation of Usitalinpimarane*



occurring of an example of diterpenoids from 8,16-cyclopimarane, *ent*-kaurane-3-oxo-16α,17-diol, in the roots of *L. usitatissimum* confirmed this proposition [8].

Compound **2** [9][10] was isolated as a light-brown amorphous powder. The HR-EI-MS gave the molecular ion peak at 114.0425, indicating the molecular formula as $C_4H_6N_2O_2$, very similar the one of maleimide-5-oxime. The ^{1}H- and ^{13}C-NMR, DEPT, and HSQC spectra of compound **2** also showed the element composition and chemical shift values of H- and C-atoms very similar to those of maleimide-5-oxime, except that the signals of the C=C bond in maleimide-5-oxime were replaced by a $-CH_2-CH_2-$ unit in **2**. The *doublet*-of-*triplet* signal of CH_2(4) at δ(H) 3.19 (*td*, $J = 6.9$, 2.4, 2 H) showed a correlation with the signal at δ(H) 7.46 (br. *s*, NH) and 2.42 (*t*, $J = 6.9$, CH_2(3)) in the ^{1}H,^{1}H-COSY spectrum as well as the NOESY experiment. The long-range correlations between δ(H) 3.19 and δ(C) 171.0, 153.9, and 30.4 and between δ(H) 2.42 and δ(C) 171.0 and 35.3 in the HMBC experiment confirmed the simple structure of **2** as (5*t*)-5-hydroxyimino)pyrrolidin-2-one, and this is the first report of the NMR data of **2**.

Three known compounds, genkwanin-4′-*O*-*β*-D-glucopyranoside [11], maleimide-5-oxime [12], and glaberide I [13], were isolated. Their structures were identified by comparison of their spectroscopic data (IR, ^{1}H- and ^{13}C-NMR, and MS) with the literature values, respectively. This is the first isolation and characterization of compound **2**, genkwanin-4′-*O*-*β*-D-glucopyranoside, and glaberide I from *L. usitatissimum*.

Hepatoprotective activities of compounds **1**, **2**, and maleimide-5-oxime against D-galactosamine-induced toxicity were examined in WB-F 344 cells. Compounds **1** and **2** were found to show a modest inhibitory activity at 10^{-4} M *in vitro* with almost the same hepatoprotective potency as that of bicyclol, a drug showing potent hepatoprotective activity [14], and without obvious cytotoxic effects (*Table 2*). This is the first report on hepatoprotective activity from the title plant.

Table 2. *Hepatoprotective Effects of Compound* **1**, **2**, *and Maleimide-5-oxime against* D-*Galactosamine-Induced Toxicity in WB-F344 cells*[a])

Compound	$x \pm s$ ($n = 3$)	Cell survival rate (100% of normal)
Normal	0.342 ± 0.083	100%
Control	0.068 ± 0.017	20%[b])
Bicyclol[c])	0.126 ± 0.025	37%[d])
1	0.131 ± 0.010	38%[c])
2	0.140 ± 0.026	41%[c])
Maleimide-5-oxime	0.100 ± 0.028	29%

[a]) Compared with normal. [b]) $P < 0.01$; compared with control. [c]) Positive control. [d]) $P < 0.05$.

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Experimental Part

General. M.p.: *XT4–100x* apparatus; uncorrected. Column chromatography (CC): silica gel (SiO_2, 200–300 mesh, *Qingdao Haiyang Chemical Group Co.*, Qingdao, P. R. China). Optical rotations: *Perkin-Elmer 241* automatic polarimeter; in $CHCl_3$ at 25°. IR Spectra: *Nicolet Impac 400* FT-IR spectrophotometer; in KBr pellets, $\tilde{\nu}$ in cm^{-1}. ^{1}H-, ^{13}C-, and 2D-NMR, and DEPT spectra: *Varian Inova-500* NMR and *Varian Mercury-300* NMR spectrometer; δ in ppm rel. to Me_4Si as internal standard, J in Hz. ESI-MS: *Agilent 100* series *LC/MSD Trap* mass spectrometer. HR-EI- and EI-MS: *England VG ZAB-HS* mass spectrometer at 70eV; in m/z (rel. %). Single-crystal X-ray crystallography: *MAC DIP-2030K*.

Plant Material. The roots of *L. usitatissimum* were collected from Longjing County, Jilin Province, P. R. China, in September 2006, and were authenticated by Prof. *Ma Lin* of the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. A voucher specimen (No. 337-11) was deposited with the Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Ministry of Education, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, P. R. China.

Extraction and Isolation. The air-dried and powdered roots (70 kg) of *L. usitatissimum* were extracted three times under reflux with H_2O. The combined H_2O extracts were evaporated under reduced pressure to about 21 l. The residue was digested in about 65 l of 95% EtOH and after letting stand for 24 h, the mixture was filtered. Evaporation of the aq. EtOH soln. under reduced pressure yielded a brown residue (1.65 kg), which was chromatographed over SiO_2 (200–300 mesh, 3.3 kg), eluted

with AcOEt, AcOEt/95% EtOH 1:1, and 95% EtOH, resp., to give three corresponding fractions, *Frs. A1 – A3*.

Fr. A1 (120 g) was subjected to CC (SiO_2, 200–300 mesh), eluting with a mixture of $CHCl_3$/MeOH of increasing polarity (100:1 → 50:1 → 25:1 → 15:1 → 10:1 → 5:1 → 0:100). Eluate obtained from $CHCl_3$/MeOH 100:1 (23.1g) was rechromatographed by CC (SiO_2, 200–300 mesh) and further eluted with a mixture of petroleum ether (PE; 60–90°)/AcOEt of increasing polarity (100:1 → 50:1 → 25:1 → 15:1 → 10:1 → 7:1 → 6:1 → 5:1 → 3:1 → 2:1 → 1:1), and the eluate obtained from PE/AcOEt 6:1 was crystallized from AcOEt to give prisms of usitatissimin A (**1**) (16 mg). The eluate obtained from $CHCl_3$/MeOH 10:1 was rechromatographed by CC (SiO_2, 200–300 mesh) and eluted with a mixture of $CHCl_3$/MeOH of increasing polarity (100:1 → 50:1 → 25:1 → 15:1 → 10:1 → 5:1 → 0:100), and genkwanin-4′-*O*-*β*-D-glucopyranoside was obtained as a yellow powder from the eluate of $CHCl_3$/MeOH 10:1.

Fr. A2 (400 g) was subjected to CC (SiO_2, 200–300 mesh), eluting with a mixture of $CHCl_3$/MeOH of increasing polarity (100:1 → 50:1 → 25:1 → 15:1 → 10:1 → 5:1 → 0:100), and compound **2** was obtained as a light-brown powder from the eluate of $CHCl_3$/MeOH 25:1, and maleimide-5-oxime was obtained as a brown powder from the eluate of $CHCl_3$/MeOH 10:1.

The air-dried and powdered roots (100 kg) of *L. usitatissimum* were extracted three times under reflux with H_2O. The combined H_2O extracts were evaporated under reduced pressure to about 35 l. The residue was extracted with about 35 l of PE (60–90°) once. The H_2O soln. was chromatographed over macroporous resin (*HP20*) eluted with H_2O, and 40, 60, and 95% EtOH, resp., to obtain four corresponding fractions, *Frs. B1 – B4*. *Fr. B2* was evaporated under reduced pressure to obtain about 520 g of residue, and 400 g of the residue was rechromatographed by CC (SiO_2, 200–300 mesh, 800 g), eluted with AcOEt, AcOEt/95% EtOH 1:1, and 95% EtOH, resp., to obtain three corresponding fractions, *Frs. B2.1 – B2.3*.

Fr. B2.1 (80 g) was subjected to CC (SiO_2, 200–300 mesh), eluting with a mixture of $CHCl_3$/MeOH of increasing polarity (100:0 → 100:1 → 50:1 → 25:1 → 15:1 → 10:1 → 5:1 → 0:100). The eluate obtained from $CHCl_3$/MeOH 100:0 and 100:1 (18.2 g) was rechromatographed by CC (SiO_2, 200–300 mesh) and further eluted with a mixture of PE (60–90°)/AcOEt of increasing polarity (100:1 → 50:1 → 25:1 → 15:1 → 10:1 → 9:1 → 8:2 → 7:3 → 6:4 → 1:1), and the eluate obtained from PE/AcOEt 7:3 was crystallized from AcOEt to give colorless crystals of glaberide I (18.2 mg).

Data of Usitatissimin A (*=(3α,5β,8α,10α,14α)-3-Hydroxy-8,16-epoxy-14,15-cyclopimar-9(11)-en-12-one*; **1**). Colorless prisms. M.p. 154.6–155.3°. $[\alpha]_D^{20} = -94.5$ ($c = 0.055$, $CHCl_3$). IR: 3512, 3244, 3014, 2973, 2939, 2841, 1698, 1637, 1408, 1469, 1374, 1089, 1047, 1014, 889, 875. ^{1}H- and ^{13}C-NMR: *Table 1*. EI-MS: 316 (M^+), 301 ($[M-Me]^+$), 298 ($[M-H_2O]^+$), 288 ($[M-CO_2]^+$). ESI-MS (pos.): 633 ($[2M+1]^+$), 655 ($[2M+Na]^+$). HR-EI-MS: 316.2032 (M^+, $C_{20}H_{28}O_3^+$; calc. 316.2038).

Data of Pyrrolidine-2,5-dione-5-oxime (*=(5E)-5-(Hydroxyimino)pyrrolidin-2-one*; **2**). Amorphous light-brown powder. ^{1}H-NMR (300 MHz, (D_6)DMSO): 9.91 (br. *s*, OH); 7.46 (br. *s*, NH); 2.42 (*t*, $J = 6.9$, CH_2(3)); 3.19 (*td*, $J = 6.9$, 2.4, CH_2(4)). ^{13}C-NMR (75 MHz, (D_6)DMSO): 171.0 (C(2)); 153.9 (C(5)); 35.3 (C(4)); 30.4 (C(3)). EI-MS: 114 (M^+), 83, 71, 57. ESI-MS (pos.): 137 ($[M+Na]^+$). HR-EI-MS: 114.0425 (M^+, $C_4H_6N_2O_2^+$; calc. 114.0429).

X-Ray Crystal-Structure Determination of **1**. Crystal data: $C_{20}H_{28}O_3$, $M_r = 316.42$, orthorhombic, space group $P2_12_12_1$, $a = 7.315(1)$, $b = 10.684(1)$, $c = 22.553(1)$ Å; $V = 1762.6(1)$ Å^3, $Z = 4$, $D_c = 1.260$ g/cm^3, $\mu = 0.09$ mm^{-1}. Intensity data were collected with *MAC DIP-2030K* image plate diffractometer with a graphite monochromator (ω-2θ scans, $2\theta_{max} = 50.0°$), MoK_α ($\lambda = 0.71073$ Å) radiation. A total of 3786 unique reflections were collected, of which 3377 were observed ($|F|^2 \geq 2\sigma|F|^2$). The structure was solved by direct methods and expanded by difference *Fourier* techniques with SHELX-97, refined on F^2 by successive full matrix least-squares techniques for non-H-atoms. H-Atoms were fixed at calculated positions. The final indices were $R_1 = 0.0404$, $wR_2 = 0.1048$, $S = 1.056$. The crystallographic data have been deposited with the *Cambridge Crystallographic Data Centre* as deposition No. CCDC-694034. Copies of the data can be obtained free of charge on application to the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44(1223)336033, or e-mail: deposit@ccdc.cam.ac.uk).

Protective Effect on Cytotoxicity Induced by D-*Galactosamine in WB-F344 Cells*. The hepatoprotective effects of compounds **1**, **2**, and maleimide-5-oxime were determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay in WB-F344 cells. Each cell suspension

of 1×10^4 cells in 100 µl of *Dulbecco*'s modified Eagles medium containing fetal calf serum (10%), penicillin (100 units/ml), and streptomycin (100 µg/ml) was planted in a 96-well microplate and precultured for 24 h at 37° under a 5% CO_2 atmosphere. Fresh medium (100 µl) containing bicyclol and test samples was added, and the cells were cultured for 1 h. Then, the cultured cells were exposed to 50 mM D-galactosamine for 24 h. Cytotoxic effects of test samples were measured simultaneously in the absence of D-galactosamine. The medium was changed into a fresh one containing 0.5 mg/ml MTT. After 4 h incubation, the medium was removed and 150 µl of DMSO was added to dissolve formazan crystals. The optical density (OD) of the formazan soln. was measured on a microplate reader at 492 nm. Inhibition [%] was obtained by the following formula:

$$\text{inhibition}[\%] = \frac{OD_{(\text{sample})} - OD_{(\text{control})}}{OD_{(\text{normal})} - OD_{(\text{control})}} \times 100$$

Statistical Analysis. All values were expressed as ± SD. The *Student*'s t-test for unpaired observation between normal or control and tested samples was carried out to identify statistical differences; p values less than 0.05 were considered as significantly different.

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