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Bioactive Sesquiterpenoids from the Rhizomes of *Acorus calamus*

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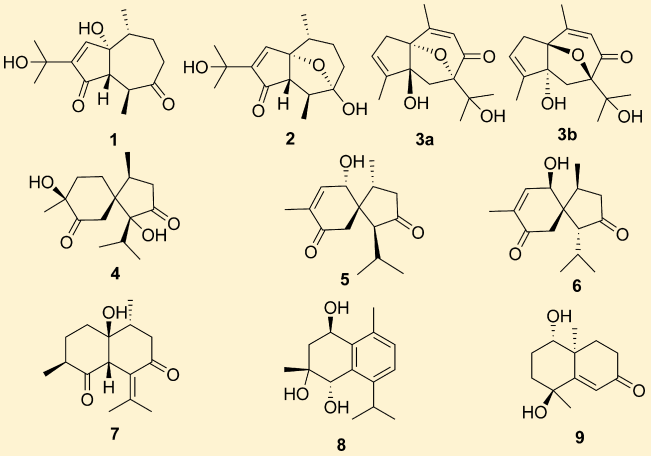
Supporting Information

ABSTRACT: Eight new sesquiterpenes (**1**–**8**) and one new norsesquiterpene (**9**) named calamusins A–I were isolated from the ethanol extract of *Acorus calamus* rhizomes. The absolute configuration of compound **8** was determined by comparing its experimental and calculated ECD spectra. The absolute configurations of the other compounds were determined from their CD spectra. Furthermore, in in vitro assays, compounds **3**, **4**, **7**, and **9** (10 μM) exhibited weak hepatoprotective activities against APAP-induced HepG2 cell damage.



Acorus calamus Linn. is commonly known as "sweet flag" and widely distributed among the northern temperate and subtropical regions of Asia, North America, and Europe. In China, it is mainly distributed in Liao Ning, Hu Bei, Hu Nan, and Si Chuan Provinces. It has been used as a traditional Chinese medicine for more than 1500 years to treat phlegm syncope, stroke, epilepsy, and rheumatism.[1] It has been reported to possess extensive antidiabetic,[2,3] nootropic,[4,5] antioxidant,[6,7] antimicrobial,[8] anti-inflammatory,[9] antispasmodic,[10] antiulcer,[11] and hypolipidemic activities.[12,13] In addition, it contains a wide variety of sesquiterpenoids, phenylpropanoids, lignans, flavonoids, steroids, and triterpenoid saponins.[14] Recently, we reported the chemical constituents of *A. tatarinowii*, which belongs to the same genus, as well as their glucokinase-activating activity.[15] As part of our ongoing research on the genus *Acorus*, the ethanol extract of dried rhizomes of *A. calamus* was investigated and eight new sesquiterpenes and one new norsesquiterpene were isolated. Furthermore, their cytotoxic and hepatoprotective activities are reported herein.

RESULTS AND DISCUSSION

The EtOH extract of dried rhizomes of *A. calamus* was suspended in water and partitioned successively with petroleum ether, EtOAc, and *n*-BuOH. The EtOAc fraction was subjected repeatedly to column chromatography on silica gel, reversed-phase C_{18} silica gel, and preparative and semipreparative HPLC to afford nine new compounds, calamusins A–I (**1**–**9**).

Calamusin A (**1**) was assigned the molecular formula $C_{15}H_{22}O_4$ on the basis of HRESIMS (*m*/*z* 289.1421 [M + Na]$^+$) and NMR data. The IR spectrum showed the absorptions of hydroxy (3425 and 3336 cm^{-1}) and carbonyl (1696 cm^{-1}) groups. The ^{1}H NMR spectrum showed signals corresponding to protons of two secondary and two tertiary methyl groups as well as one olefinic proton. ^{13}C NMR and DEPT-135/90 spectra exhibited signals corresponding to 15 carbons, including four methyl, two secondary, four tertiary, and five quaternary carbons. The chemical shifts indicated that **1** contained a carbonyl, an α,β-unsaturated carbonyl, a double bond, and two oxygenated quaternary carbons. The structural units of C-4–C-5–C-6–C-15 and C-1–C-2–C-14 were determined from the ^{1}H–^{1}H COSY spectrum. The HMBC correlations from H-12/H-13 to C-9/C-11 indicated the existence of a dimethylcarbinol moiety located at C-9. Additionally, the HMBC correlations from Me-15 to C-7 and correlations from H-1 to C-3/C-6/C-7 allowed the definition of the cycloheptanone unit. The $J_{1,2}$ value of 11.0 Hz indicated that these two protons are *trans*-oriented. Furthermore, NOESY (performed in DMSO-d_6) correlations of H-1 with Me-14/H-4a and H-4a (β-oriented) with H-6 suggested H-1 and Me-14 are β-oriented and Me-15 and 7-OH are α-oriented. The CD spectrum of **1** showed a negative Cotton effect at 327 nm ($\Delta\varepsilon$ −1.12), due to the n→π^* transition of the α,β-unsaturated carbonyl moiety.[16] Therefore, the absolute configuration of **1** was determined to be 1*S*,2*S*,6*R*,7*S*.

Calamusin B (**2**) was determined to have the same molecular formula as **1** by HRESIMS and NMR data. The IR spectrum

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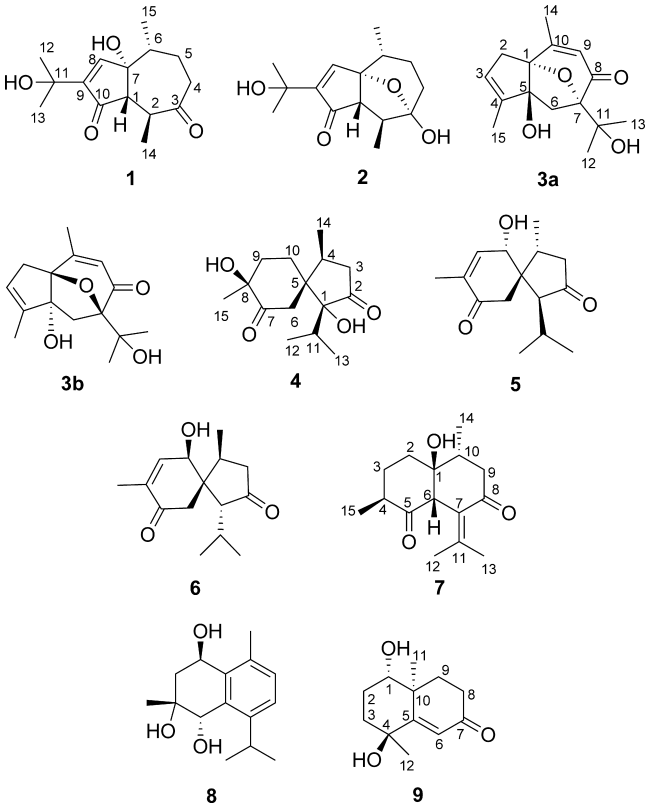

exhibited absorptions of hydroxy (3406 cm^{-1}) and carbonyl (1702 cm^{-1}) groups. The ^1H and ^{13}C NMR data were similar to those of **1** except that the chemical shift of C-3 was shifted from δ 213.7 to 110.7. Therefore, we speculated that a hemiacetal substructure was formed at C-3, which was later confirmed by 2D NMR. Combined analyses of the degrees of unsaturation and chemical shifts verified that an oxygen bridge is formed between C-3 and C-7. The coupling constant (J = 10.0 Hz) between β-oriented H-1 and H-2 indicated that H-2 is α-oriented. The NOESY correlation between H-1 and H-6 suggested H-6 is β-oriented and that Me-15 is α-oriented. The absolute configuration of **2** was determined to be 1*S*,2*S*,3*S*,6*R*,7*S* on the basis of the negative Cotton effect observed in its CD spectrum at 339 nm ($\Delta\varepsilon$ −0.69).[16] Comparison of compounds **2** and **1** suggested that **2** was probably formed from **1** during the silica gel chromatographic procedure.

The molecular formula of calamusin C (**3**) was determined to be $C_{15}H_{20}O_4$ by HRESIMS (m/z 265.1434 $[M + H]^+$) and NMR spectra. The ^1H NMR spectrum showed signals corresponding to the protons of four methyl groups and two olefinic protons. ^{13}C NMR and DEPT-135/90 spectra displayed 15 signals corresponding to four methyl, two methylene, two olefinic methine, two olefinic quaternary, and four oxygenated quaternary carbons as well as an α,β-unsaturated carbonyl carbon. On the basis of the number of oxygenated carbons, an oxygen bridge was present in the structure. The $^1H-^1H$ COSY correlations between H-2 and H-3 and between H-3 and Me-15, as well as the HMBC correlations from Me-15 to C-3/C-4/C-5 and correlations from H-2 to C-1/C-3/C-4/C-5, suggested the existence of the cyclopentene partial structure, and Me-15 is attached at C-4. The $^1H-^1H$ COSY correlations between Me-14 and H-9, as well as the HMBC correlations from Me-14 to C-1/C-9/C-10, suggested the existence of the partial structure C-1−C-10(C-14)−C-9. The HMBC correlations from Me-12/Me-13 to C-7/C-11 indicated the presence of a dimethylcarbinol moiety, which is attached to C-7. The HMBC correlations from H-6 to C-7/C-8/C-11 indicated the presence of the partial structure C-6−C-7−C-8. Analysis of the chemical shifts and degrees of unsaturation indicated that the oxygen bridge connected C-1 and C-7. The ROESY correlations of OH-5 with Me-14/H-6a (β), and H-6b (α) with Me-12/Me-13, determined that OH-5 is β-oriented and the oxygen bridge is α-oriented. The low specific rotation (−3.2) and the lack of distinct Cotton effects in the CD spectrum indicated that **3** comprised a racemic mixture. Chiral HPLC analysis (Figure 1) and the CD spectra (Figure 2) of the resolved enantiomers (**3a** and **3b**) confirmed

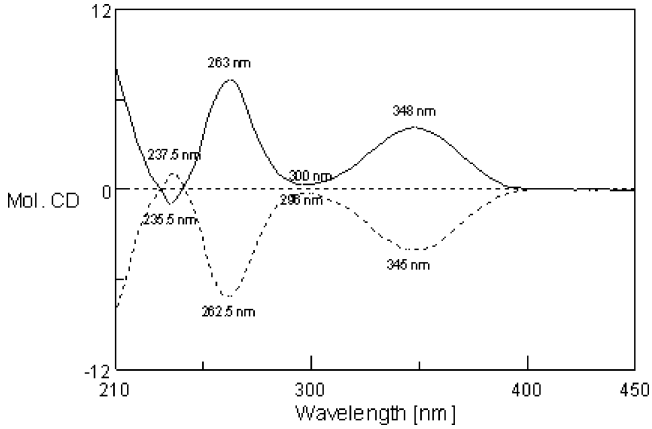

Figure 2. ECD spectra of compounds **3a** (solid line) and **3b** (dashed line) in MeOH.

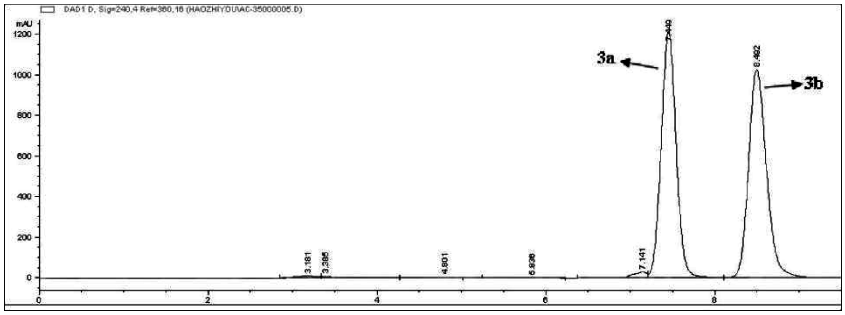

Figure 1. HPLC profiles of compound **3** [column, Daicel Chiralpak AD-H (5 μm, 4.6 × 250 mm); mobile phase, *n*-hexane/2-propanol = 9:1; flow rate: 1 mL/min; UV detection at 240 nm; peak area of **3a**:**3b** = 1:1].

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that **3** was a racemic mixture. The configuration of **3a** was determined to be 1*R*,5*S*,7*S* on the basis of the positive Cotton effect that was observed in its CD spectrum at 348 nm ($\Delta\varepsilon$ +4.11),[16] and the configuration of **3b** was determined to be 1*S*,5*R*,7*R*.

On the basis of HRESIMS (*m*/*z* 291.1568 [M + Na]$^+$) and NMR spectra, the molecular formula of calamusin D (**4**) was determined to be $C_{15}H_{24}O_4$. The IR spectrum showed bands corresponding to hydroxy (3494 and 3442 cm^{-1}) and carbonyl (1736 and 1711 cm^{-1}) groups. The ^{1}H NMR spectrum exhibited signals corresponding to the protons of a tertiary and three secondary methyl groups. ^{13}C NMR and DEPT-135/90 spectra displayed 15 signals corresponding to two carbonyl, four methyl, four methylene, two methine, and two oxygenated quaternary carbons as well as one quaternary aliphatic carbon. The combined analyses of chemical shifts and degrees of unsaturation indicated that **4** has a bicyclic sesquiterpenoid skeleton. The ^{1}H−^{1}H COSY spectrum showed correlations corresponding to three partial structures: C-3−C-4−C-14, C-9−C-10, and a C-12−C-11−C-13 isopropyl group. HMBC correlations from Me-14 to C-5, H-3 to C-1/C-2/C-5, and H-11 to C-1/C-5 indicated the presence of a cyclopentanone substructure and implied that the isopropyl group is linked to C-1. HMBC correlations from Me-15 to C-7/C-8/C-9, H-6 to C-5/C-7/C-8/C-10, H-9 to C-5, and H-10 to C-5/C-6 indicated the presence of a cyclohexanone moiety. In addition, HMBC correlations from H-4 to C-10, H-10 to C-1/C-4, and H-6 to C-1/C-4/C-5 showed the connection of two cyclic moieties to C-5. The NOESY correlations between Me-14 and H-11/H-3a (β), Me-12 and H-6a (β), H-4 and H-9b (β), and H-9a (α) and H-10a (α)/H-6b/Me-15 determined the relative configuration of **4**. The cyclohexanone moiety provides stronger contributions to the CD spectrum than those of the cyclopentanone moiety in this type of acorane sesquiterpene.[17] Thus, we analyzed the spectrum based on the cyclohexanone moiety. According to the octant rule of cycloketones,[18] the negative Cotton effect that occurred at 306 nm ($\Delta\varepsilon$ −2.91) based on the n→π* transition of cyclohexanone indicated that the configuration of compound **4** is 1*R*,4*S*,5*S*,8*S*.

Calamusin E (**5**) was assigned the molecular formula $C_{15}H_{22}O_3$ on the basis of HRESIMS (*m*/*z* 273.1465 [M + Na]$^+$) and NMR data. The UV spectrum (236 nm) indicated the presence of an α,β-unsaturated carbonyl moiety. The IR spectrum showed bands corresponding to hydroxy (3324 cm^{-1}) and carbonyl (1715, 1672 cm^{-1}) groups. The ^{1}H NMR spectrum displayed signals corresponding to the protons of three secondary methyl groups, an olefinic methyl group, a trisubstituted double bond, and an oxygenated methine group. ^{13}C NMR and DEPT-135/90 spectra demonstrated 15 signals corresponding to four methyl, two methylene, and three aliphatic methine carbons as well as an oxygenated methine carbon, a trisubstituted double bond, a carbonyl carbon, an α,β-unsaturated carbonyl carbon, and a quaternary carbon at δ 55.2 (C-5), which is a characteristic signal of acorane-type sesquiterpenoids. The ^{1}H−^{1}H COSY correlations suggested the presence of C-15−C-8−C-9−C-10 and C-14−C-4−C-3 moieties. The HMBC correlations from Me-14 to C-3 and from H-3 to C-2 and C-5 suggested the presence of the cyclopentanone moiety. In addition, HMBC correlations from H-6 to C-5/C-7/C-8/C-10, H-10 to C-8/C-9, H-9 to C-5/C-7/C-15, and Me-15 to C-7/C-8/C-9 indicated the presence of a cyclohexanone moiety. HMBC correlations from H-1/H-4 to C-5/C-10, H-6 to C-1/C-4/C-5, and H-10 to C-1/C-4 demonstrated the presence of two cyclic moieties linked together at C-5. NOESY correlations between Me-14 and H-10, H-6, and Me-13 indicated that the isopropyl group is β-oriented, whereas OH-10 and Me-14 are α-oriented. The negative Cotton effect that occurred at 335 nm ($\Delta\varepsilon$ −0.38) and is based on the n→π* transition of an α,β-unsaturated cyclohexanone unit, indicated that the configuration of **5** is 1*S*,4*R*,5*R*,10*S*.[16] Additionally, according to the helicity rule,[19] the positive Cotton effect observed at 248 nm ($\Delta\varepsilon$ +9.32) and is based on the π→π* transition of an α,β-unsaturated cyclohexanone moiety, also supports the above conclusion.

Calamusin F (**6**) was determined to have the same molecular formula as **5** on the basis of the HRESIMS and NMR spectra. The UV, IR, ^{1}H NMR, and ^{13}C NMR spectroscopic data were similar to those of **5**. Thus, we speculated that **6** possesses the same planar structure as **5**, and 2D NMR (^{1}H−^{1}H COSY, HSQC, and HMBC) confirmed this conclusion. The relative configuration of **6** was determined by the NOESY correlations between Me-14 and H-1/H-6b (β)/3b (β), H-1 and H-6a/6b, and H-6a and H-10. In the structure of **6**, the carbonyl and double bond of the α,β-unsaturated cyclohexanone system are almost within the same plane. Thus, the Cotton effect of saturated cyclopentanone was used to analyze the configuration of **6**. The negative Cotton effect observed at 290 nm ($\Delta\varepsilon$ −1.55) in the CD spectrum, according to the n→π* transition of saturated cyclopentanone,[18] indicated that the absolute configuration of **6** is 1*R*,4*S*,5*R*,10*R*.

The molecular formula of calamusin G (**7**), $C_{15}H_{22}O_3$, was obtained by HRESIMS (*m*/*z* 251.1644 [M + H]$^+$) and NMR spectra. The characteristic UV signal of an α,β-unsaturated carbonyl group was observed at 250 nm (MeOH, log ε 3.67). The IR spectrum indicated the presence of hydroxy (3350 cm^{-1}) and carbonyl (1703, 1670 cm^{-1}) groups. A comparison of the ^{1}H and ^{13}C NMR data indicated that **7** possesses the same gross structure as the reported compound, (1*R*,4*R*,6*S*,10*R*)-1-hydroxy-7(11)-cadinen-5,8-dione, which was also isolated from *A. calamus*.[20] The 2D NMR data (^{1}H−^{1}H COSY, HSQC, and HMBC) confirmed the above conclusion. The NOESY correlations between Me-15 and H-6/H-3b, H-3a, and Me-14 indicated that H-6 and Me-15 are β-oriented and Me-14 is α-oriented. In addition, the NOESY correlation between H-6 and Me-12 suggested that the two were *cis*-oriented in the C-7−C-11 double-bond system. The configuration analysis indicated that either OH-1 is not α-oriented or Me-14 is too distant from H-3α to generate an NOE signal. Additionally, a 1D NOE experiment (acetone-d_6) demonstrated that irradiation of H-6 caused an increase of the OH-1 resonance and irradiation of OH-1 caused an increase of the H-6 resonance. Thus, OH-1 was determined to be β-oriented. Owing to the presence of two cyclohexanone moieties in this structure, the Cotton effects observed in the CD spectrum, which are based on the n→π* transition of cyclohexanones, may interact. On the basis of the helicity rule,[19] the positive Cotton effect (248 nm, $\Delta\varepsilon$ +4.40) observed, originating from the π→π* transition of the conjugated carbonyl group, indicated that the absolute configuration of **7** is 1*S*,4*S*,6*S*,10*R*.

Calamusin H (**8**) was assigned the molecular formula $C_{15}H_{22}O_3$ on the basis of HRESIMS (*m*/*z* 273.1470 [M + Na]$^+$) and NMR spectra. The ^{1}H NMR and ^{13}C NMR data of **8** were similar to those of *cis*-4,5-dihydroxycorocalane,[21] even though C-2 is oxygenated. Analysis of the HMBC spectra and molecular formula verified the planar structure of **8**. The NOESY correlations of Me-15 with H-5/H-3b and H-3a with

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H-2 indicated that H-5/H-15/H-3b/OH-2 are β-oriented and that H-3a/H-2/OH-4/OH-5 are α-oriented. The absolute configuration of **8** was determined by comparing experimental and calculated ECD spectra. The latter was performed with time-dependent density functional theory. Optimized geometries were obtained by system conformational analysis using the MMFF94 force field, and ECD spectra were calculated in methanol solution using the B3LYP/6-31g(d) level of theory with the CPCM model. The results showed that the experimental and calculated spectra were in good agreement (Figure 3). Thus, the absolute configuration of **8** was determined to be 2*R*,4*R*,5*S*.

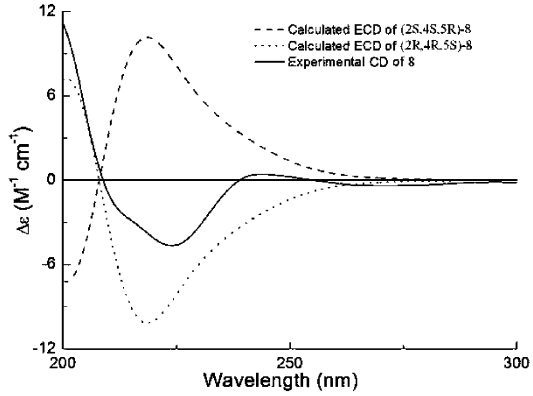

Figure 3. Calculated and experimental ECD spectra of compound **8** in MeOH.

Calamusin I (**9**) was deduced to have the molecular formula $C_{12}H_{18}O_3$ by HRESIMS (*m*/*z* 211.1324 $[M + H]^+$) and NMR spectra. The ^{1}H NMR spectrum displayed signals corresponding to two tertiary methyl groups, an olefinic proton, and an oxygenated methine proton. ^{13}C NMR and DEPT-135/90 spectra exhibited 12 signals corresponding to two methyl and four methylene carbons, an α,β-unsaturated carbonyl carbon, a trisubstituted double bond, an oxygenated methine carbon, an oxygenated quaternary carbon, and an aliphatic quaternary carbon. The ^{1}H–^{1}H COSY spectrum indicated the presence of C-1–C-2–C-3 and C-9–C-8 moieties. The HMBC correlations from H-2 to C-3/C-4 and H-3 to C-4/C-5 suggested that C-4 is linked with C-3 and C-5, the correlations from Me-12 to C-3/C-4/C-5 indicated that Me-12 is attached to C-4, the correlations from Me-11 to C-1/C-5/C-10 suggested that Me-11 is linked with C-10 and that C-10 is linked with C-1 and C-5, and the correlations from H-6 to C-7/C-8 and from H-8 to C-9/C-10 confirmed the existence of an α,β-unsaturated cyclohexanone substructure. The NOESY correlations between Me-12 and H-3a (α), H-3b (β) and H-1, H-1 and H-9b (β), and H-9a (α) and Me-11 determined that OH-1, Me-11, and Me-12 are α-oriented. The positive Cotton effect (340 nm, +0.23) observed in the CD spectrum, which is based on the n→π* transition of an α,β-unsaturated cyclohexanone moiety, indicated that the absolute configuration of **9** is 1*S*,4*S*,10*S*.[16]

Compounds **1**–**9** were tested for their cytotoxicity against five human tumor cell lines, HCT-8 (human ileocecal adenocarcinoma cell line), Bel-7402 (human hepatoma cell line), BGC-823 (human gastric cancer cell line), A549 (human lung epithelial cell line), and A2780 (human ovarian cancer cell line); however, they were inactive (IC_{50} >10 μM). They were also bioassayed for their hepatoprotective activities against *N*-acetyl-*p*-aminophenol (APAP)-induced toxicity in HepG2 (human hepatocellular liver carcinoma cell line) cells, using the hepatoprotective activity drug bicyclol as the positive control.[22] As shown in Figure 4, compounds **3**, **4**, **7**, and **9** exhibited moderate hepatoprotective activities.

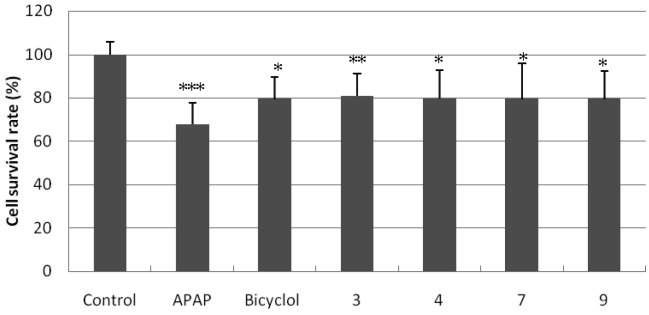

Figure 4. Hepatoprotective effects of compounds **3**, **4**, **7**, and **9** (10 μM) against APAP-induced toxicity in HepG2 cells. Results are expressed as the mean ± SD (n = 3). Bicyclol was used as positive control (10 μM). ***p < 0.001; *p < 0.05; **p < 0.01.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a JASCO P-2000 polarimeter. UV spectra were recorded on a JASCO V-650 spectrophotometer, and IR spectra were recorded on a Nicolet 5700 spectrometer using a FT-IR microscope transmission method. NMR spectra were acquired with INOVA-500 and Bruker AV500-III spectrometers. HRESIMS spectra were collected on an Agilent 1100 series LC/MSD ion trap mass spectrometer. ECD spectra were recorded on a JASCO J-815 spectropolarimeter. Melting points were measured on an XT5B micromelting point apparatus and are uncorrected. Analytical HPLC was performed on an Agilent 1200 Infinity system with Agilent Zorbax SB-C18 (5 μm, 4.6 × 150 mm) and Daicel Chiralpak AD-H columns (5 μm, 4.6 × 250 mm). Semipreparative HPLC was performed on a Shimadzu LC-6AD pump with a Shimadzu SPD-M20A detector and an Agilent Zorbax SB-C18 column (5 μm, 9.4 × 250 mm). Column chromatography was performed on silica gel (200–300 mesh, Qingdao Marine Chemical Factory, China) and ODS (50 μm, YMC, Japan), respectively. GF254 plates were used for TLC (Qingdao Marine Chemical Factory, China).

Plant Material. The rhizomes of *A. calamus* were purchased from Anguo county, Hebei Province, China, and identified by Professor Lin Ma from the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. A voucher specimen (no. ID-S-2281) is deposited at the herbarium of the Institute of Material Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, China.

Extraction and Isolation. The dried rhizomes of *A. calamus* (19.8 kg) were smashed and extracted with 95% ethanol (120 L × 2 h × 3). The concentrated extract (3.1 kg) was suspended in H_2O (3 L) and partitioned with petroleum ether (PE), EtOAc, and *n*-BuOH (3 L × 3), successively. The EtOAc extract (310 g) was subjected to a silica gel column (10.5 × 100 cm) and eluted with CH_2Cl_2/MeOH (40:1, 20:1, 15:1, 10:1, 8:1, 6:1, 5:1, 3:1, 1:1, and 0:1 v/v) to produce 10 fractions (F1–F10). F4 (76 g) was subjected to a silica gel column (10 × 60 cm) and eluted with PE/acetone (7:1, 5:1, 4:1, 3:1, 2:1, and 0:1 v/v) to produce eight fractions (F4a–F4h). F4c (26 g) was subjected to a silica gel column (5 × 60 cm) and eluted with PE/acetone (4:1, 2:1, and 1:1 v/v) to yield five fractions (F4c1–F4c5). F4c2 (9 g) was chromatographed on a reversed-phase C_{18} silica gel column (4.5 × 42 cm) and eluted with MeOH/H_2O (30:70, 50:50, 70:30, and 100:0 v/v) to yield eight fractions (F4c2a–F4c2h). F4c2c (620 mg) was purified by using preparative HPLC (16% MeCN/H_2O, 4 mL/min) to yield compound **1** (9.4 mg, t_R 155 min). F4c2d (330 mg) was purified by using semipreparative HPLC (27% MeCN/H_2O, 2.5 mL/min) to

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Table 1. ^{1}H NMR Data of Compounds 1−9 (500 MHz)

position	1[a]	1[b]	2[c]	3[c]	3[d]	4[c]
1	2.47, d (11.0)	2.25, d (11.0)	2.87, d (10.0)			
2	2.71, m	2.44, overlap	2.40, m	*β*. 2.91, m	*β*. 2.90, m	
				α. 2.35, m	*α*. 2.28, m	
3				5.49, m	5.44, brs	*β*. 2.47, dd (19.0, 10.0)
						α. 2.03, dd (19.0, 6.0)
4	*β*. 2.74, m	*β*. 2.66, m	a. 1.91, m			2.25, m
	α. 2.51, m	*α*. 2.45, overlap	b. 1.60, overlap			
5	*α*. 2.07, m	*α*. 1.91, m	a. 2.09, m			
	β. 1.73, m	*β*. 1.45, m	b. 1.58, overlap			
6	1.86, m	1.67, m	1.70, m	*β*. 2.69, d (13.5)	*β*. 2.72, d (14.0)	*β*. 3.15, d (13.0)
				α. 1.57, d (13.5)	*α*. 1.61, d (14.0)	*α*. 2.43, dd (13.0, 2.0)
8	7.24, s	7.25, s	7.42, s			
9				5.99, dd (2.5, 1.5)	5.96, d (1.5)	*α*. 1.95, m
						β. 1.73, m
10						*α*. 2.38, m
						β. 0.87, m
11						2.14, m
12	1.49, s	1.26, s[e]	1.35, s	1.31, s	1.25, s	1.02, d (6.5)
13	1.49, s	1.25, s[e]	1.35, s	1.16, s	1.07, s	0.75, d (7.0)
14	1.40, d (6.5)	1.14, d (6.5)	0.94, d (7.5)	2.06, d (1.5)	2.07, d (1.5)	1.21, d (7.5)
15	1.16, d (6.5)	1.00, d (6.5)	1.21, d (6.5)	1.80, m	1.78, dd (4.0, 1.5)	1.21, s
OH		5.30, brs (OH-7)			4.19, s (OH-5)	
OH		4.97, brs (OH-11)			3.44, brs (OH-11)	

position	5[c]	6[c]	7[c]	8[c]	9[d]
1	2.32, overlap	1.98, m			3.39, m
2			2.10, m	4.94, dd (4.0, 2.0)	1.80, m
3	a. 2.39, dd (12.5, 8.0)	*α*. 2.73, q (10.0)	*α*. 1.82, m	*α*. 2.24, dd (15.0, 4.5)	*α*. 1.85, dt (9.0, 3.0)
	b. 1.89, overlap	*β*. 1.72, m	*β*. 1.72, m	*β*. 1.97, dt (15.0, 1.5)	*β*. 1.70, m
4	2.32, overlap	2.52, m	2.52, m		
5				4.55, d (1.0)	
6	2.53, d (2.5)	*α*. 2.63, d (16.0)	4.12, s		6.25, d (1.0)
		β. 2.53, d (16.0)			
8				7.22, d (8.0)	*α*. 2.44, m
					β. 2.20, m
9	6.68, m	6.64, s	*α*. 2.34, dd (16.5, 11.5)	7.13, d (8.0)	*α*. 2.25, m
			β. 2.24, dd (16.5, 5.5)		*β*. 1.75, m
10	4.54, dd (4.5, 2.0)	4.67, t (2.0)	1.88, m		
11	1.89, overlap	2.02, ddd (9.5, 7.0, 2.5)		3.44, m	1.27, d (1.0)
12	1.07, d (8.5)	1.16, d (7.0)	1.68, s	1.23, d (7.5)	1.37, d (1.0)
13	0.81, d (6.5)	1.42, d (7.0)	2.07, s	1.28, d (7.0)	
14	1.04, d (7.0)	0.94, d (7.5)	0.91, d (6.5)	2.38, s	
15	1.75, m	1.77, t (1.5)	1.30, d (7.5)	1.40, s	
OH					4.03, brs (OH-1)
OH					3.93, brs (OH-4)

[a]In $CDCl_3$. [b]In DMSO-d_6. [c]In methanol-d_4. [d]In acetone-d_6. [e]Signals may be reversed in the same column.

yield compound **6** (3.4 mg, t_R 35 min). Fraction F4b (7 g) was chromatographed on a reversed-phase C_{18} silica gel column (4.5 × 42 cm) and eluted with MeOH/H_2O (30:70, 50:50, 70:30, and 100:0 v/v) to yield seven fractions (F4b1−F4b7). F4b2 was purified by using semipreparative HPLC (5% MeCN/H_2O, 2.5 mL/min) to yield compound **9** (8.8 mg, t_R 41 min). F4b3 was purified by using semipreparative HPLC (17% MeCN/H_2O, 2.5 mL/min) to produce compounds **2** (7.3 mg, t_R 54 min), **4** (7.1 mg, t_R = 66 min), and **7** (3.2 mg, t_R 61 min). F4b4 was purified by using semipreparative HPLC (19% MeCN/H_2O, 2.5 mL/min) to yield compounds **3** (5.3 mg, t_R 33 min) and **5** (7.5 mg, t_R 71 min). Compound **3** was isolated by using a chiral analytical column (90% *n*-hexane/2-propanol, 1 mL/min) to yield compounds **3a** (0.56 mg, t_R 7.5 min) and **3b** (0.53 mg, t_R 8.5 min). F4b5 was chromatographed by using semipreparative HPLC (35% MeOH/H_2O, 2.5 mL/min) to yield compound **8** (2.7 mg, t_R 88 min).

*Calamusin A (**1**):* pale yellow oil ($CHCl_3$); $[\alpha]^{20}_D$ +15.3 (*c* 0.24, MeOH); UV (MeOH) λ_{max} (log ε) 218 (3.68), 202 nm (4.01); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 327 (−1.12), 296 (−1.27), 234 nm (+5.98); IR ν_{max} 3425, 3336, 2975, 1696, 1457, 1315, 1168 cm^{-1}; ^{1}H (500 MHz, $CDCl_3$ and DMSO-d_6) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Tables 1 and 2; HRESIMS *m*/*z* 267.1603 $[M + H]^+$ (calcd for $C_{15}H_{23}O_4$, 267.1596), 289.1421 $[M + Na]^+$ (calcd for $C_{15}H_{22}O_4Na$, 289.1416).

*Calamusin B (**2**):* colorless oil (MeOH); $[\alpha]^{20}_D$ +36.1 (*c* 0.24, MeOH); UV (MeOH) λ_{max} (log ε) 221 (3.73), 202 nm (3.81); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 339 (−0.69), 249 (+0.68), 232 (+0.09), 205 nm (+3.66); IR ν_{max} 3406, 2973, 2936, 1702, 1461, 1312, 1113, 959 cm^{-1}; for ^{1}H (500 MHz, methanol-d_4) and ^{13}C NMR (125 MHz, methanol-d_4) data, see Tables 1 and 2; HRESIMS (positive ion mode) *m*/*z* 289.1409 $[M + Na]^+$ (calcd for $C_{15}H_{22}O_4Na$, 289.1416).

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Table 2. ^{13}C NMR Data of Compounds 1−9 (125 MHz)

position	1[a]	2[b]	3[b]	3[c]	4[b]	5[b]	6[b]	7[b]	8[b]	9[c]
1	63.7	58.8	93.3	92.8	86.0	36.1[d]	61.0	76.8	135.7	78.3
2	44.7	41.3	36.0	35.8	219.2	220.3	220.1	31.9	66.8	28.7
3	213.7	110.7	126.6	125.8	41.8	46.0	47.2	27.4	37.5	39.3
4	41.7	33.4	142.2	142.1	33.0	36.0[d]	32.1	44.9	73.5	71.7
5	29.2	28.0	96.5	96.2	53.3	55.2	55.3	214.4	71.6	174.8
6	38.7	33.9	37.9	37.8	41.3	39.8	45.7	61.2	134.3	124.3
7	80.3	86.7	93.1	92.3	214.3	200.5	200.9	128.7	148.2	199.3
8	155.6	152.2	201.1	not obsd	75.1	136.2	136.3	203.8	126.5	34.1
9	148.9	156.6	127.2	126.7	37.0	149.9	151.0	46.2	131.3	38.5
10	206.1	206.9	168.4	167.2	28.2	70.6	71.9	34.2	136.3	41.8
11	69.7	70.4	73.0	72.3	32.6	28.1	25.8	150.7	28.9	18.2
12	28.7	28.4	25.9	26.0	18.6	24.5	24.9	22.6	25.4	30.5
13	28.7	28.2	24.4	24.2	19.2	19.0	18.9	23.8	24.2	
14	16.2	11.9	22.3	22.3	19.3	15.3	18.7	14.5	19.0	
15	18.0	14.0	12.5	12.5	24.0	15.2	15.0	17.5	27.1	

[a]In $CDCl_3$. [b]In methanol-d_4. [c]In acetone-d_6. [d]Signals may be reversed in the same column.

*Calamusin C (**3**, racemic mixture):* colorless oil (MeOH); $[\alpha]^{20}_D$ −3.2 (*c* 0.26, MeOH); UV (MeOH) λ_{max} (log ε) 242 (3.73), 202 nm (3.83); IR ν_{max} 3408, 2977, 2925, 1663, 1440, 1378, 1183, 1113 cm^{-1}; ^{1}H (500 MHz, methanol-d_4 and acetone-d_6) and ^{13}C NMR (125 MHz, methanol-d_4 and acetone-d_6) data, see Tables 1 and 2; HRESIMS *m*/*z* 265.1434 [M + H]$^+$ (calcd for $C_{15}H_{21}O_4$, 265.1440), 287.1258 [M + Na]$^+$ (calcd for $C_{15}H_{20}O_4Na$, 287.1259). (+)-Calamusin C (**3a**): colorless oil (MeOH); $[\alpha]^{20}_D$ +321(*c* 0.03, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 348 (+4.11), 300 (+0.29), 263 (+7.27), 236 nm (−1.00). (−)-Calamusin C (**3b**): colorless oil (MeOH); $[\alpha]^{20}_D$ −317.6 (*c* 0.03, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 345 (−4.01), 296 (−0.24), 263 (−7.16), 238 nm (+1.05).

*Calamusin D (**4**):* colorless, needle-type crystals (MeOH); mp 111−114 °C; $[\alpha]^{20}_D$ −67.8 (*c* 0.24, MeOH); UV (MeOH) λ_{max} (log ε) 203 nm (3.67); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 306 (−2.91), 205 nm (+3.36); IR ν_{max} 3494, 3442, 2981, 2945, 1736, 1711, 1389, 1280, 1198 cm^{-1}; ^{1}H (500 MHz, methanol-d_4) and ^{13}C NMR (125 MHz, methanol-d_4) data, see Tables 1 and 2; HRESIMS *m*/*z* 291.1568 [M + Na]$^+$ (calcd for $C_{15}H_{24}O_4Na$, 291.1572), 269.1744 [M + H]$^+$ (calcd for $C_{15}H_{25}O_4$, 269.1753).

*Calamusin E (**5**):* colorless needle-type crystals (MeOH); mp 67−69 °C; $[\alpha]^{20}_D$ +154.3 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (logε) 236 (3.57), 202 nm (3.85); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 335 (−0.38), 299 (+2.13), 248 (+9.32), 209 nm (+7.74); IR ν_{max} 3468, 3324, 2965, 2935, 1715, 1672, 1651, 1456, 1384, 1265 cm^{-1}; ^{1}H (500 MHz, methanol-d_4) and ^{13}C NMR (125 MHz, methanol-d_4) data, see Tables 1 and 2; HRESIMS *m*/*z* 251.1643 [M + H]$^+$ (calcd for $C_{15}H_{23}O_3$, 251.1647), 273.1465 [M + Na]$^+$ (calcd for $C_{15}H_{22}O_3Na$, 273.1467).

*Calamusin F (**6**):* colorless oil (MeOH); $[\alpha]^{20}_D$ −74.0 (*c* 0.11); UV (MeOH) λ_{max} (log ε) 234 (3.06), 202 nm (4.03); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 321 (−0.40), 290 (−1.55), 271 (−1.26), 245 (−2.89), 211 nm (+3.06); IR ν_{max} 3443, 2962, 2926, 1730, 1676, 1462, 1385, 1365, 1093 cm^{-1}; ^{1}H (500 MHz, methanol-d_4) and ^{13}C NMR (125 MHz, methanol-d_4) data, see Tables 1 and 2; HRESIMS *m*/*z* 251.1639 [M + H]$^+$ (calcd for $C_{15}H_{23}O_3$, 251.1647), 273.1462 [M + Na]$^+$ (calcd for $C_{15}H_{22}O_3Na$, 273.1467).

*Calamusin G (**7**):* colorless, needle-type crystals (MeOH); mp 161−163 °C; $[\alpha]^{20}_D$ +33.7 (*c* 0.22, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.77), 250 nm (3.67); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 295.5 (−2.19), 248 (+4.40), 212.5 nm (−1.01); IR ν_{max} 3350, 2943, 1703, 1670, 1619, 1462, 1245, 989 cm^{-1}; ^{1}H (500 MHz, methanol-d_4) and ^{13}C NMR (125 MHz, methanol-d_4) data, see Tables 1 and 2; HRESIMS *m*/*z* 251.1644 [M + H]$^+$ (calcd for $C_{15}H_{23}O_3$, 251.1647), 273.1463 [M + Na]$^+$ (calcd for $C_{15}H_{22}O_3Na$, 273.1467).

*Calamusin H (**8**):* white, amorphous powder (MeOH); $[\alpha]^{20}_D$ −9.4 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.29), 219 nm (3.83); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 224 (−2.33), 243 (+0.21), 272 nm (−0.20); IR ν_{max} 3321, 2960, 1463, 1444, 1031, 1011 cm^{-1}; ^{1}H (500 MHz, methanol-d_4) and ^{13}C NMR (125 MHz, methanol-d_4) data, see Tables 1 and 2; HRESIMS *m*/*z* 273.1470 [M + Na]$^+$ (calcd for $C_{15}H_{22}O_3Na$, 273.1467).

*Calamusin I (**9**):* colorless oil (MeOH); $[\alpha]^{20}_D$ +34.9 (*c* 0.29, MeOH); UV (MeOH) λ_{max} (log ε) 241 (3.96), 201 nm (3.70); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 340 (+0.23), 294 (−0.03), 243.5 (+1.34), 221 (−0.12), 105 nm (+1.27); IR ν_{max} 3446, 2938, 1654, 1463, 1412, 1232, 1157, 1039 cm^{-1}; ^{1}H (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) data, see Tables 1 and 2; HRESIMS *m*/*z* 211.1324 [M + H]$^+$ (calcd for $C_{12}H_{19}O_3$, 211.1334), 233.1144 [M + Na]$^+$ (calcd for $C_{12}H_{18}O_3Na$, 233.1154).

Cytotoxicity Testing. Cell proliferation inhibition was determined using the MTT method.[23,24]

Hepatoprotective Activity Assay. Human HepG2 hepatoma cells were cultured in DMEM medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 + 95% air. The cells were then passaged by treatment with 0.25% trypsin in 0.02% EDTA. The MTT assay was used to assess the cytotoxicity of test samples.[22] The cells were seeded in 96-well multiplates. After an overnight incubation at 37 °C with 5% CO_2, 10 μM test samples and APAP (final concentration of 8 mM) were added into the wells and incubated for another 48 h. Then, 100 μL of 0.5 mg/mL MTT was added to each well after the withdrawal of the culture medium and incubated for an additional 4 h. The resulting formazan was dissolved in 150 μL of DMSO after aspiration of the culture medium. The plates were placed on a plate shaker for 30 min and read immediately at 570 nm using a microplate reader.

■ ASSOCIATED CONTENT

Supporting Information

MS, 1D NMR, 2D NMR, and CD spectra for compounds **1**−**9** are 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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