Four Novel Metabolites from Microbial Transformation of Curcumol by *Cunninghamella blakesleana*

Hui ZHANG,[a] Ning KANG,[b] Feng QIU,*[,a] Ge-Xia QU,[a] and Xin-Sheng YAO[a]

[a] *Department of Natural Products Chemistry, Shenyang Pharmaceutical University; Shenyang, 110016, P. R. China: and* [b] *Department of Biochemistry and Molecular Biology, Shenyang Pharmaceutical University; Shenyang, 110016, P. R. China.* Received July 24, 2006; accepted November 28, 2006

Further study on the microbial transformation of curcumol (1) by *Cunninghamella blakesleana* (AS3.970) led to the isolation of four novel metabolites. Their structures were elucidated as 3β-hydroxy curcumol (2), 12-hydroxy curcumol (3), 1α-hydroxy-10β,14-epoxy curcumol (4) and (2*S*,4*S*,5*S*,7*S*)-10-hydroxymethyl-7-isopropyl-2-methoxy-4-methyl-1-oxaspiro[4,6]undec-10-en-8-one (5) on the basis of spectral methods. All of them were characterized as new compounds.

Key words curcumol; microbial transformation; metabolite; *Cunninghamella blakesleana*

Curcuma wenyujin was used in traditional Chinese medicine for the treatment of various cancers such as cervical carcinoma, vulva cancer, skin neoplasm, thyroid tumor, esophageal neoplasm, gastric and intestinal cancer,[1] and its essential oil was currently embodied in the Pharmacopoeia of the P. R. China (2005), as an anti-cancer and anti-virus remedy.[2] It showed that the Ezhu intravenous injection made from *C. wenyujin* has high anti virus activity in treating respiratory syncytial virus (RSV) in infant therapy.[3,4] Curcumol, one of the major components of the essential oil with the structure of sesquiterpene hemiacetal, was found to have obvious anti-tumor activity.[5] The structure of curcumol was identified on the basis of chemical and spectral data in 1965,[6] and its stereostructure was determined by X-ray analysis in 1984.[7] As an anti-tumor and anti-virus agent, curcumol has the disadvantages of poor solubility in water and rather strong toxicity (ID_{50} of rats is 250 mg/kg). Thus, further structural modifications of curcumol to generate new analogs with increased water solubility, improved bioactivity and less toxic, may provide high utility in cancer and other diseases treatment.

Up to now, structural modifications of curcumol by biological methods have not been performed. The species of *Cunninghamella* have been reported to be able to catalyze specific hydroxylation of substrates.[8,9] In our previous study, we used *Cunninghamella blakesleana* (AS 3.970) to transform curcumol, and yielded six metabolites.[10] Further study led to the isolation of other four novel metabolites. In this paper, we'll describe the isolation and determination of the four novel metabolites.

Results and Discussion

Curcumol (**1**) was administered to the 2-d-old microorganism cultures, and four more polar metabolites were obtained after additional 5 d of incubation. On the basis of physicochemical evidences and the spectroscopic analysis, their structures were identified as: 3β-hydroxy curcumol (**2**), 12-hydroxy curcumol (**3**), 1α-hydroxy-10β,14-epoxy curcumol (**4**) and (2*S*,4*S*,5*S*,7*S*)-10-hydroxymethyl-7-isopropyl-2-methoxy-4-methyl-1-oxaspiro[4,6]undec-10-en-8-one (**5**) (see Fig. 1). The ^{13}C- and ^{1}H-NMR spectral data were assigned using 2D-NMR techniques in Tables 1 and 2.

The HR-ESI-MS analysis of compound **2** (*m*/*z* 253.1796 $[M+H]^+$) indicated the molecular formula $C_{15}H_{24}O_3$ in combination with the ^{1}H- and ^{13}C-NMR spectral data, which had one more oxygen than that of the substrate molecule, revealing that **2** might be a hydroxylated product of curcumol. In the ^{1}H-NMR spectrum, an additional signal assigned to H-3 was observed at δ 4.09 (1H, br s) in contrast with that of **1**. The ^{13}C-NMR spectrum of **2** exhibited an additional oxygen-bearing methine signal at δ 74.9, suggesting the introduction of a hydroxyl group in the molecule. Its location was deduced to be at C-3 by the long-range coupling from 15-methyl protons (δ 1.06, 3H, d, J=7.1 Hz) to C-3 in the HMBC spectrum. Meanwhile the signals of C-2 and C-4 had shifted downfield from δ 28.2 to δ 41.0 and from δ 39.4 to δ 44.6, and the signals of C-1 and C-15 had shifted upfield from δ 54.5 to δ 52.7 and from δ 12.3 to δ 6.6, in comparison with that of **1**. The β-configuration of 3-OH was deduced from the presence of the correlation between H-3 (δ 4.09)

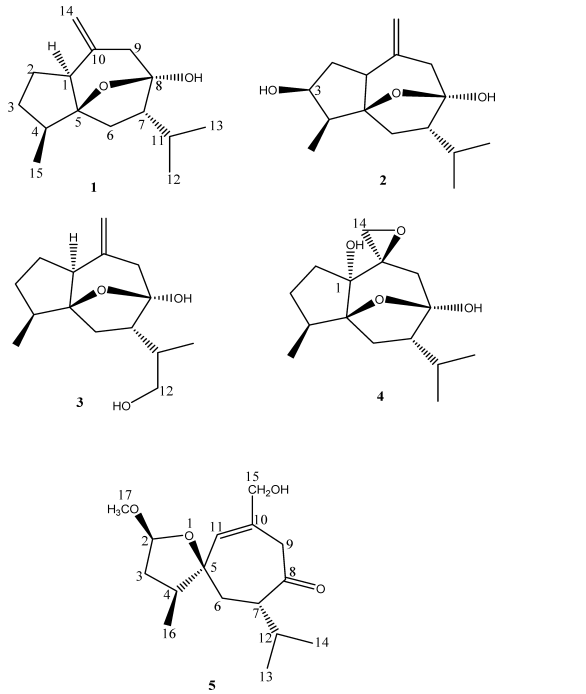

Fig. 1. The Structures of Curcumol (**1**) and Its Biotransformation Metabolites (**2**—**5**)

* To whom correspondence should be addressed. e-mail: fengqiu2000@tom.com

Table 1. ^{1}H-NMR Data of Curcumol (**1**) and Its Metabolites

Proton	**1**[a)]	**2**[a)]	**3**[b)]	**4**[c)]	**5**[b)]
1	2.18 (1H, t, J=9.4 Hz)	2.10 (1H, t, J=10.1 Hz)	2.21 (1H, dd, J=10.9, 9.2 Hz)		
2	1.65 (2H, m)	1.59 (1H, m) 2.42 (1H, m)	1.68 (2H, m)	1.45 (1H, m) 2.15 (1H, m)	4.98 (1H, dd, J=5.1, 6.3 Hz)
3	1.47 (1H, m) 1.90 (1H, m)	4.09 (1H, br s)	1.45 (1H, m) 1.96 (1H, m)	1.35 (1H, m) 1.69 (1H, m)	1.66 (1H, ddd, J=11.8, 5.1, 1.6 Hz) 2.46 (1H, m)
4	1.88 (1H, m)	1.77 (1H, m)	1.88 (1H, m)	2.28 (1H, m)	2.15 (1H, m)
6	1.18 (1H, dd, J=12.5, 6.7 Hz) 2.14 (1H, t, J=12.5 Hz)	1.18 (1H, dd, J=12.6, 6.8 Hz) 2.18 (1H, dd, J=12.6, 12.3 Hz)	1.24 (1H, dd, J=12.6, 6.4 Hz) 2.12 (1H, dd, J=12.6, 11.8 Hz)	1.93 (2H, m)	1.96 (1H, br s) 1.98 (1H, br s)
7	1.47 (1H, m)	1.45 (1H, m)	1.72 (1H, m)	1.69 (1H, m)	2.54 (1H, m)
9	2.51 (1H, d, J=14.6 Hz) 2.58 (1H, d, J=14.6 Hz)	2.52 (1H, dd, J=15.0 Hz) 2.60 (1H, dd, J=15.0 Hz)	2.47 (1H, br, J=14.7 Hz)	1.83 (1H, d, J=13.5 Hz) 2.18 (1H, d, J=13.5 Hz)	2.66 (1H, d, J=14.0 Hz) 3.65 (H, dd, J=14.0, 2.9 Hz)
11	1.73 (1H, m)	1.69 (1H, m)	1.71 (1H, m)	1.69 (1H, m)	5.39 (1H, br s)
12	1.01 (3H, d, J=6.2 Hz)	1.01 (3H, d, J=6.4 Hz)	3.24 (1H, dd, J=10.7, 6.5 Hz) 3.52 (1H, dd, J=10.7, 2.9 Hz)	0.93 (3H, d, J=5.9 Hz)	2.18 (1H, m)
13	0.87 (3H, d, J=6.4 Hz)	0.87 (3H, d, J=6.4 Hz)	1.06 (3H, d, J=6.1 Hz)	1.00 (3H, d, J=5.9 Hz)	0.89 (3H, d, J=6.8 Hz)
14	4.88 (2H, br s)	4.89 (3H, d, J=6.5 Hz) 4.91 (1H, d, J=2.2 Hz)	4.86 (2H, br s)	2.56 (1H, d, J=6.2 Hz) 3.25 (1H, dd, J=6.2, 1.4 Hz)	0.97 (3H, d, J=7.0 Hz)
15	1.00 (3H, d, J=6.5 Hz)	1.06 (3H, d, J=7.1 Hz)	0.97 (3H, d, J=6.6 Hz)	0.95 (3H, d, J=6.9 Hz)	3.92 (1H, br d, J=12.0 Hz) 3.95 (1H, br d, J=12.0 Hz) 1.03 (3H, d, J=7.0 Hz) 3.34 (3H, s)

Spectra were recorded at 400 MHz NMR in *a*) $CDCl_3$, *b*) CD_3OD or *c*) $(CD_3)_2CO$. Chemical shifts are expressed in ppm downfield from TMS.

Table 2. ^{13}C-NMR Data of Curcumol (**1**) and Its Metabolites

Carbon	**1**[a)]	**2**[a)]	**3**[b)]	**4**[c)]	**5**[b)]
1	54.5	52.7	55.9	80.5	
2	28.2	41.0	29.2	33.9	106.7
3	30.9	74.9	32.0	29.8	41.2
4	39.4	44.6	40.6	37.3	46.4
5	88.1	88.5	89.0	89.1	87.2
6	34.7	34.6	35.0	30.1	29.6
7	56.5	56.2	50.7	56.6	53.2
8	104.5	105.1	105.7	105.1	213.5
9	38.8	38.9	39.8	40.8	43.8
10	144.7	143.9	146.5	60.4	136.4
11	28.7	28.8	37.7	30.8	130.6
12	21.5	21.6	68.0	21.6	32.3
13	23.1	23.2	16.3	23.7	19.2
14	112.9	114.0	113.3	55.3	20.8
15	12.3	6.62	12.7	12.5	67.9 13.5 56.6

Spectra were recorded at 400 MHz NMR in *a*) $CDCl_3$, *b*) CD_3OD or *c*) $(CD_3)_2CO$. Chemical shifts are expressed in ppm downfield from TMS.

and H-4 (δ 1.77) in the NOESY spectrum. Thus, **2** was characterized as 3β-hydroxy curcumol.

The molecular formula of metabolite **3** was determined to be $C_{15}H_{24}O_3$ from its HR-ESI-MS analysis (*m/z* 253.1794 $[M+H]^+$) and ^{1}H- and ^{13}C-NMR spectral data, which is the same as that of **2**, indicating **3** might be a hydroxylated product of **1**. Its ^{1}H-NMR, in contrast with that of **1**, showed the disappearance of a methyl group at δ 0.87 and the presence of a new $-CH_2OH$ group, which resonated as a pair of doublets at δ 3.24 (J=10.7, 6.5 Hz) and δ 3.52 (J=10.7, 2.9 Hz). This indicated that a methyl group had been oxidized to a methylol during the incubation. Meanwhile the ^{13}C-NMR spectrum showed the C-12 signal at δ 21.5 was replaced by a new signal at δ 68.0 in comparison with that of **1**, indicating the methyl group at C-12 has been oxized to a hydroxymethyl group. And consequently, the signal of C-11 had shifted downfield from δ 28.7 to δ 37.7 and the signals of C-13 and C-7 had shifted upfield from δ 23.0 to δ 16.3 and from δ 56.5 to δ 50.7. Furthermore, the long-range coupling from 13-methyl protons (δ 1.06, 3H, d, J=6.1 Hz) to C-12 in the HMBC spectrum also conformed the above conclusion. The configuration of C-11 was established to be *R* based on the NOESY spectrum, in which H-12 correalted with H-6, H-11 correlated with H-9, and H-13 correlated with H-9. Therefore, the structure of **3** was identified as 12-hydroxy-(11*R*)-curcumol.

The HR-ESI-MS of metabolite **4** showing a quasimolecular ion peak at *m/z* 269.1739 ($[M+H]^+$), together with the NMR data suggested the molecular formula $C_{15}H_{24}O_4$, which had two more oxygen than that of the substrate molecule, suggesting that **4** might be a dihydroxylated product of curcumol. In contrast with that of **1**, the ^{1}H-NMR spectrum of **4** showed the disappearance of H-1 (δ 2.18) and H-14 (δ 4.88) in **1**, indicating the location of oxidation at C-1 and C-14. Its ^{13}C-NMR spectrum, compared with that of **1**, showed the disappearance of the olefinic signals at δ 112.9 and 144.7 in **1** and the occurrence of two oxidized carbon signals at δ 55.3 (C-14) and 60.4 (C-10) in **4**, revealing the formation of an epoxy ring between C-10 and C-14. Meanwhile, the signal of C-1 had shifted downfield from δ 54.5 to δ 80.5 due to the deshielding effect of hydroxyl group. The correlations from H-9 (δ 1.83, 1H, d, J=13.5 Hz; δ 2.18, 1H, d, J=13.5 Hz) to C-1, C-10 and C-14 in the HMBC spectrum also supported

the above deduction. The relative stereochemistry of **4** was confirmed by NOESY experiment: 1-OH (δ 3.54, 1H, s) correlated with H-4 (δ 2.28, 1H, m) suggesting the α-configuration of 1-OH. The signal at δ 1.83 (H-9) correlated with 7-isopropyl (δ 1.00, 3H, d, J=5.9 Hz, H-12) which has α-configuration indicating the configuration of this proton as α. The correlations between H-14a (δ 2.56, 1H, d, J=6.2 Hz) and H-9α, H-14b (δ 3.25, 1H, dd, J=6.2, 1.4 Hz) and 1-OH suggested the α-configuration of C-14. Thus, the structure of metabolite **4** was identified as 1α-hydroxy-10β,14-epoxy curcumol. The proton and carbon NMR spectra data were assigned in Tables 1 and 2 on the basis of 2D-techniques.

The HR-ESI-MS analysis of metabolite **5** (*m*/*z* 283.1905 $[M+H]^+$) and the NMR data (Tables 1, 2) revealed the molecular formula as $C_{16}H_{26}O_4$. Its ^{1}H-NMR spectrum showed three methyl signals at δ 0.89 (3H, d, J=6.8 Hz, H-13), δ 0.97 (3H, d, J=7.0 Hz, H-14) and δ 1.03 (3H, d, J=7.0 Hz, H-16) in the upfield, which were very similar with those of the substrate. In the downfield of the ^{1}H-NMR, three groups of proton signals at δ 3.34 (3H, s, H-17), δ 3.92 (1H, br d, J=12.0 Hz, H-15a), δ 3.95 (1H, br d, J=12.0 Hz, H-15b) and δ 4.98 (1H, dd, J=5.1, 6.3 Hz, H-2), and an olefinic proton signal at δ 5.39 (1H, br s, H-11) were observed. The ^{13}C-NMR spectrum displayed a carbonyl signal at δ 213.5 (C-8), two olefinic carbon signals at δ 136.4 (C-10) and δ 130.6 (C-11), an acetal carbon signal at δ 106.7 (C-2) and three oxidized carbon signals at δ 87.2 (C-5), δ 67.9 (C-15) and δ 56.6 (C-17) in the downfield. Based on the analysis of the DEPT135 and HSQC spectra, three methyl carbon signals at δ 13.5 (C-16), δ 19.2 (C-13) and δ 20.8 (C-14), three methylene carbon signals at δ 29.6 (C-6), δ 41.2 (C-3) and δ 43.8 (C-9) and three methine carbon signals at δ 32.3 (C-12), δ 46.4 (C-4) and δ 53.2 (C-7) in the upfield could be assigned. In the HMBC spectrum, correlations from H-16 (δ 1.03), H-2 (δ 4.98), H-3α (δ 2.46) and H-4 (δ 2.15) to C-5 (δ 87.2), and from H-3β (δ 2.15) and H-4 to C-16 (δ 13.5) indicated the skeleton structure of ring A as 4-methyl-tetrahydrofuran, which was also demonstrated by the correlations of H-16 with H-4, H-4 with H-3, H-3 with H-2 in the ^{1}H–^{1}H COSY spectrum. The correlations from H-17 (δ 3.34) to C-2 (δ 106.7) in the HMBC spectrum suggested the methoxyl group was linked to C-2. Correlations from H-6 (δ 1.96, δ 1.98), H-7 (δ 2.54), H-9 (δ 2.66, δ 3.65) and H-12 (δ 2.18) to C-8 (δ 213.5), from H-15 (δ 3.92, δ 3.95), H-11 (δ 5.39) and H-7 to C-9 (δ 43.8), from H-13 (δ 0.89) and H-14 (δ 0.97) to C-12 (δ 32.3), and from H-6 to C-5 in the HMBC spectrum revealed the structure of ring B as 10-hydroxymethyl-7-isopropyl-10-en-8-one, which was attached to ring A at C-5 to form a spiro-ring. This conclusion was further supported by the HMBC correlations from H-6 to C-4 and C-11, from H-11 to C-6 and C-4, and from H-4 to C-6 and C-11. All the ^{1}H- and ^{13}C-NMR data were assigned according to extensive NMR techniques (DEPT, ^{1}H–^{1}H COSY, HSQC and HMBC) (see Tables 1 and 2) and the configuration of **5** was established by the NOESY spectrum (see Fig. 2). In conclusion, the structure of metabolite **5** was elucidated as (2*S*,4*S*,5*S*,7*S*)-10-hydroxymethyl-7-isopropyl-2-methoxy-4-methyl-1-oxaspiro[4,6]undec-10-en-8-one.

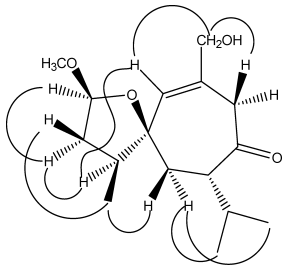

Fig. 2. The NOESY Correlations of Metabolite **5**

Experimental

IR spectra were conducted on a Bruker IFS 55 spectrophotometer (KBr). Optical rotation values were measured by using a Perkin-Elmer 243B polarimeter. NMR spectra (^{1}H-, ^{13}C-NMR, DEPT, ^{1}H–^{1}H-COSY, HSQC, HMBC and NOESY) were recorded in $CDCl_3$, $(CD_3)_2CO$ or CD_3OD on Bruker AVANCE-400 spectrometer (^{1}H-NMR, 400 MHz; ^{13}C-NMR, 100 MHz) and chemical shifts were recorded in ppm using TMS as internal standard. ESI-MS spectra were measured on a Bruker esquire 2000 in the positive mode. HPLC was carried out on Waters 600 (Waters, U.S.A.) with PDA996 (Waters, U.S.A.) as the detector, using the reversed phase column (Techsphere C_{18}, 5 μm, 4.6×250 mm, for analysis and Inertsil C_{18} Prepare column 20.0×250 mm, for preparation). Silica gel for column chromatography and TLC were obtained from Qingdao Oceanic Chemical Factory. All chemicals were obtained from Shenyang Chemical Factory.

Microorganisms *Cunninghamella blakesleana* (AS3.970) which were purchased from China General Microbiological Culture Collection Center, was gifted from Dr. Dean Guo.

Substrate Material Curcumol **1** (99% as the pureness, detected with HPLC) was isolated from the essential oil of *Curcuma wenyujin*, and characterized by chemical and spectral methods as described in the literature.[11] The substrate was dissolved in acetone and diluted to 4.0 mg/ml before use.

Medium All culture and biotransformation experiments were performed in potato medium, which was produced by the following procedure: 200 g of minced husked potato were boiled in water for an hour, then the extract was filtered and the filtrate were added with water to 1 l after addition of 20 g of glucose.

Biotransformation The screen-scale biotransformation was performed in 250 ml Erlenmeyer flasks containing 60 ml potato media. Twenty-nine kinds of microorganisms were transferred into the flasks from the slants respectively. The cultures were cultivated on rotary shakers at 180 rpm, 25 °C. One milliliter of the substrate solution was added into each flask with 2-d-old microorganism cultures, and 1 ml of acetone alone instead of substrate solution into each parallel flask as the culture control. Substrate controls consisted of sterile media containing the same amount of substrate and incubated under the same conditions. After further 5 d of incubation, the broth was filtered under vacuum and the filtrate was extracted with the equivalent volume of ethyl acetate for three times. The dried mycelium were extracted with acetone (3×50 ml) at room temperature and filtered under vacuum. All the extract were pooled respectively and evaporated under reduced pressure at 45 °C to give residue. Then the residue was dissolved in methanol and spotted on silica gel plates which were developed by cyclohexane–acetone (2 : 1), and visualized by spraying with 10% H_2SO_4 (in EtOH), followed by heating at about 100 °C. For preparative biotransformation, 2 g of substrate were distributed into shake flasks with 2-d-old microorganism cultures, and after another 5 d of incubation, the culture media were collected, extracted and concentrated as described above.

Isolation and Purification of the Metabolites The obtained residue (5.6 g) was separated on a silica gel (200—300 mesh) column (ϕ35×420 mm), eluted with cyclohexane–acetone (100, 100 : 1, 100 : 3, 100 : 5, 10 : 1, 5 : 1, 2 : 1, 1 : 1, 1500 ml for each gradient eluent) to give fractions I—XII. Fraction IX (1.5 g) was subjected to silica gel column (ϕ30×450 mm) chromatography, eluted with a mixture of cyclohexane–acetone (20 : 1, 10 : 1, 5 : 1, 2 : 1, 1000 ml for each gradient eluent) to give fraction A—J, among which fraction G and H were further separated by preparative HPLC (Inertsil C_{18} Prepare column ϕ20×250 mm, 55% MeOH/H_2O as mobile phase, flow rate: 8 ml/min) to yield **2** (26.3 mg) and **3** (18.5 mg). Fraction VIII (1.0 g) was subjected to silica gel column (ϕ35×280 mm) chromatography, eluted with cyclohexane–acetone (50 : 1, 20 : 1, 10 : 1, 5 : 1, 500 ml for each gradient eluent) to yield **4** (3.5 mg) as crystals (between

1000 ml and 1500 ml). Fraction X was subjected to silica gel column (ϕ30×450 mm) chromatography, eluted with a mixture of cyclohexane–acetone (20 : 1, 10 : 1, 5 : 1, 2 : 1, 1 : 1, 1000 ml for each gradient eluent) to give fraction A—L, among which fraction D was further separated by preparative HPLC (Inertsil C_{18} Prepare column ϕ20×250 mm, 50% MeOH/H_2O as mobile phase, flow rate: 8 ml/min) to yield **5** (2.2 mg).

3β-Hydroxy Curcumol (**2**): Colorless oil; ESI-MS (*m*/*z*): 275 $[M+Na]^+$; positive HR-ESI-MS, *m*/*z*: 253.1796 $[M+H]^+$ (Calcd for $C_{15}H_{25}O_3$: 253.1804); the ^{1}H- and ^{13}C-NMR data were assigned in Tables 1 and 2.

12-Hydroxy-(11*R*)-curcumol (**3**): Colorless crystals (MeOH); IR ν_{max} (KBr): 3526.2, 2957.3, 2364.3, 1646.9, 1412.6, 1122.4, 895.8, 601.7, 472.5 cm^{-1}; ESI-MS (*m*/*z*): 275 $[M+Na]^+$; positive HR-ESI-MS, *m*/*z*: 253.1794 $[M+H]^+$ (Calcd for $C_{15}H_{25}O_3$: 253.1804); the assignments of the proton and carbon NMR spectra were summarized in Tables 1 and 2.

1α-Hydroxy-10β,14-epoxy Curcumol (**4**): Colorless crystals (acetone); IR ν_{max} (KBr): 3448.1, 2961.2, 2364.3, 1654.6, 1458.9, 1368.2, 1133.9, 990.3, 944.7, 470.5 cm^{-1}; ESI-MS (*m*/*z*): 291 $[M+Na]^+$; positive HR-ESI-MS, *m*/*z*: 269.1739 $[M+H]^+$ (Calcd for $C_{15}H_{25}O_4$: 269.1753); the proton and carbon NMR spectra data were assigned in Tables 1 and 2.

(2*S*,4*S*,5*S*,7*S*)-10-Hydroxymethyl-7-isopropyl-2-methoxy-4-methyl-1-oxaspiro[4,6]undec-10-en-8-one (**5**): Colorless crystals (MeOH); ESI-MS (*m*/*z*): 305 $[M+Na]^+$; positive HR-ESI-MS, *m*/*z*: 283.1905 $[M+H]^+$(Calcd for $C_{16}H_{27}O_4$: 283.1909); the ^{1}H- and ^{13}C-NMR data were assigned in Tables 1 and 2.

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