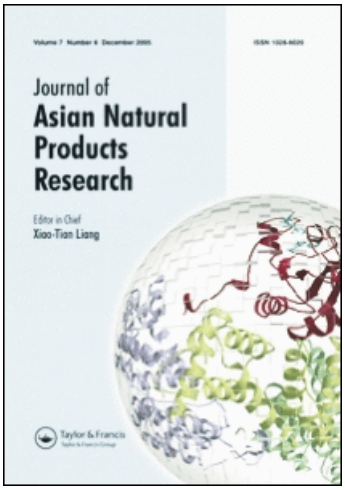

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Microbial transformation of curcumol by *Cunninghamella blakesleana*

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Microbial transformation of curcumol (**1**) by *Cunninghamella blakesleana* (AS 3.970) yielded six metabolites. On the basis of spectral data, their structures were elucidated as 14-hydroxy-9*E* curcumol (**2**), 10*R*,14-dihydroxy curcumol (**3**), 10*S*,14-epoxy curcumol (**4**), 10*R*,14-epoxy curcumol (**5**), 10*S*,14-epoxy-7,11-dehydrocurcumol (**6**), 10*R*,14-epoxy-7,11-dehydrocurcumol (**7**), respectively. Among them **2**, **3**, **6** and **7** are new compounds; **4** and **5**, **6** and **7** are two pairs of epimers.

Keywords: Microbial transformation; Curcumol; *Cunninghamella blakesleana*; Epimer

1. Introduction

Curcuma wenyujin was used in traditional Chinese medicine for the treatment of various cancers such as cervical carcinoma, vulva cancer, skin neoplasm, thyroid tumour, esophageal neoplasm, gastric and intestinal cancer [1], and its essential oil is currently used as a clinical remedy for uterus cancer [2]. The Ezhu intravenous injection made from *C. wenyujin* has been used as anti-virus medicine in recent years, especially 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 hemiketal, was found to have obvious anti-tumour 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 anti-tumour and anti-virus agents, 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 analogues 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. In this paper, the microbial transformation of curcumol by *Cunninghamella blakesleana* (AS 3.970) is reported. Six metabolites were isolated from the fermentation

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broth and their structures were elucidated on the basis of the spectral data. There were four new compounds and two pairs of epimers to be identified from the metabolites.

2. Results and discussion

Curcumol (**1**) was administered to the 2-day-old microorganism cultures, and six more polar metabolites were obtained after additional 5 days of incubation. On the basis of physicochemical evidence and the spectroscopic analysis, their structures were identified as 14-hydroxy-9*E* curcumol (**2**), 10*R*,14-dihydroxy curcumol (**3**), 10*S*,14-epoxy curcumol (**4**), 10*R*,14-epoxy curcumol (**5**), 10*S*,14-epoxy-7,11-dehydro curcumol (**6**) and 10*R*,14-epoxy-7,11-dehydrocurcumol (**7**), among which metabolites **2**, **3**, **6** and **7** are four new compounds; **4** and **5**, **6** and **7** are two pairs of epimers. The structures of **1** and metabolites were illustrated in figure 1.

Metabolite **2** was obtained as colourless crystals. Its molecular formula $C_{15}H_{24}O_3$ was established by HRFAB-MS at *m/z* 253.1796 $[M + H]^+$. It had one more oxygen than that of the substrate molecule, suggesting that **2** might be a hydroxylated product of **1**. The ^{13}C NMR and the DEPT spectra of **2** were similar to those of **1**, except for C-1, C-7, C-9 and C-14, among which the olefinic methene (C-14) had shifted downfield from δ 112.9 to 123.6 as a methine (C-9), and the methene signal at δ 38.8 (C-9) in the substrate disappeared, instead a hydroxyl methyl signal occurred at δ 64.5 (C-14) in **2**, suggesting that the double bond had transferred from C10 (14) to C-9 (10), and the hydroxylation had taken place at C-14. Meanwhile, C-1 had shifted upfield from δ 54.5 to 49.6 due to the γ-effect. The above changes were confirmed by the HMBC experiment: H-9 (δ 5.83, br.s) correlated with C-8 (δ 103.4), C-14 (δ 64.5) and C-1 (δ 49.6) (see figure 2). Therefore the structure of metabolite **2** was identified as 14- hydroxy-9*E* curcumol.

Metabolite **3** was obtained as colourless crystals, Its molecular formula $C_{15}H_{26}O_4$ was established by HRFAB-MS at *m/z* 271.1904 $[M + H]^+$. It had two more hydroxyl groups than that of **1**, suggesting that **3** might be a dihydroxylated product of **1**. Additionally, the strong absorption at 3569 and 3303 cm^{-1} in the IR spectra also supported this deduction. The ^{13}C NMR spectra of **3**, compared with that of **1**, showed that the double bond at C-10 (14) in **1** had disappeared and two hydroxylated carbons at δ 70.9 (C-14) and δ 74.9 (C-10) in **3** occurred, suggesting the double bond had been oxygenised. This conclusion was demonstrated by the HMBC experiment: H-1 (δ 1.64, br.t, $J = 11.1$ Hz), H-2a (δ 1.51, m), H-9b (δ 1.90, d, $J = 14.5$ Hz) and H-14 (δ 3.27, d, $J = 10.9$ Hz; δ 3.37, d, $J = 10.9$ Hz) correlated with C-10 (δ 74.9), as well as H-9a (δ 1.52, d, $J = 14.5$ Hz) with C-14 (δ 70.9). Meanwhile, H-9b correlated with H-11 (δ 2.30, m) in the NOESY spectra suggested H-9b as α configuration. Therefore, H-9β (δ 1.52) correlated with H-14 (δ 3.27, δ 3.37) indicated the orientation of C-14 as β-configuration. Thus, the structure of metabolite **3** was assigned to be 10*R*,14-dihydroxy curcumol.

Metabolite **4** was obtained as colourless crystals. Its molecular formula $C_{15}H_{24}O_3$ was determined by HRFAB-MS at *m/z* 253.1798$[M + H]^+$. Comparison of the ^{13}C NMR spectrum of **4** with that of **1** showed the disappearance of the olefinic carbon signals at δ 112.9 (C-14) and 144.7 (C-10) in **1** and the occurrence of two oxidised carbon signals at δ 59.7 (C-14) and 58.2 (C-10) in **4**, revealing the formation of an epoxy ring between C-10 and C-14. This compound was first obtained by Hiroshi Hikino *et al*., who oxidised curcumol

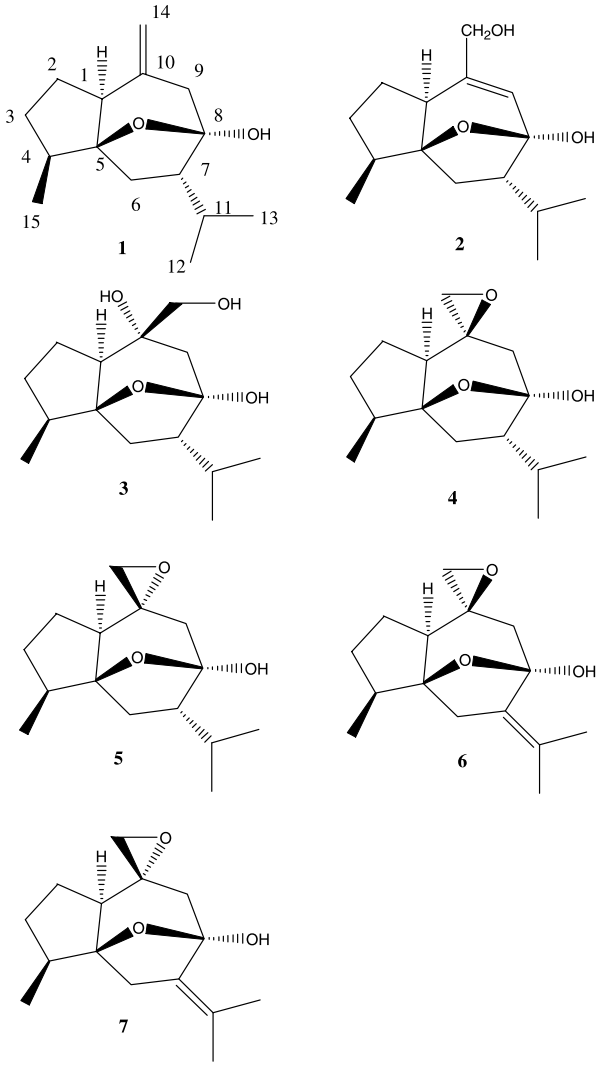


Figure 1. The absolute structures of curcumol and the metabolites.

with perbenzoic acid to yield **4** and **5** [5]. But there was no report about the stereochemistry and the assignments of the ^{1}H NMR and ^{13}C NMR data in detail. The relative stereochemistry of **4** was confirmed by NOESY experiment (see figure 3): H-6α (δ 1.36, dd, $J = 12.7$, 6.6 Hz) which correlated with H-1 (δ 1.53) and H-13 (δ 0.94, d, $J = 6.0$ Hz) in the NOESY spectrum and H-1 (δ 1.53) correlated with H-14 (δ 2.76) suggesting the α-configuration of C-14. On the basis of above analysis, the structure of metabolite **4** was identified as 10*S*, 14-epoxy curcumol.

Metabolite **5** was obtained as colourless crystals. Its molecular formula $C_{15}H_{24}O_3$ was inferred from the HRFAB-MS at *m/z* 253.1797 $[M + H]^+$. The ^{13}C NMR spectrum of **5** was very similar to that of metabolite **4** except for C-14, which had shifted upfield from δ 59.7 to 49.1 due to the field effect of the oxygen bridge between C-5 and C-8, indicating metabolite **5** might be the epimer of metabolite **4**. Therefore, the structure of metabolite **5** was concluded to be 10*R*, 14-epoxy curcumol.

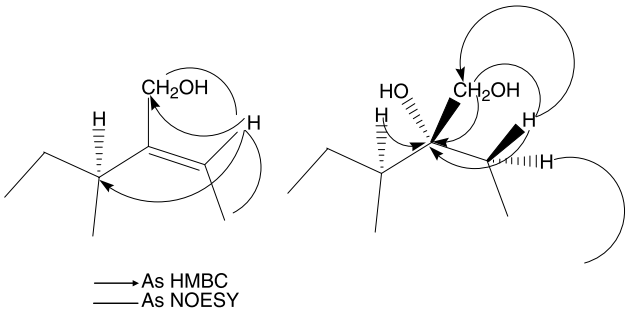
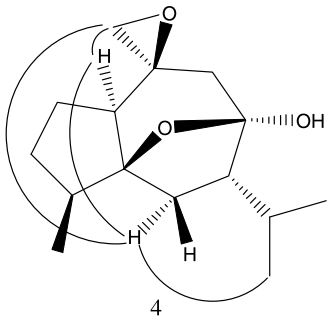


Figure 2. Key HMBC and NOESY correlations of **2** and **3**.

Metabolite **6** was obtained as colourless needles. Its molecular formula $C_{15}H_{22}O_3$ was established by HRFAB-MS at *m/z* 251.1641 $[M + H]^+$. The ^{13}C NMR spectra of **6**, in contrast with that of **4**, showed the new occurrence of two olefinic carbon signals at δ 128.0 (C-11) and 134.8 (C-7), and the disappearance of the signals of C-7 at δ 55.9 and C-11 at 29.5 in **4**, which indicated that a new double bond between C-7 and C-11 in **6** was formed. This deduction was further supported by the HMBC experiment: H-12 (δ 1.79, dd, $J = 2.3$, 1.3 Hz), H-13 (δ 1.67, br.s) and H-6a (δ 2.20, dt, $J = 15.2$, 1.0 Hz) correlated with C-11(δ 128.0) as well as H-12, H-13, H-6 and H-9b (δ 2.25, dd, $J = 12.2$, 1.4 Hz) with C-7 (δ 134.4). In conclusion, the structure of metabolite **6** was assigned to be 10*S*,14-epoxy-7, 11- dehydrocurcumol.

Metabolite **7** was obtained as colourless needles. Its molecular formula $C_{15}H_{22}O_3$ was determined by HRFAB-MS at *m/z* 251.1641 $[M + H]^+$. The ^{13}C NMR spectrum of **7** was very similar to that of metabolite **6** except for C-14, which had shifted upfield from δ 58.3 to 49.3 due to the field effect of the oxygen bridge between C-5 and C-8, indicating metabolite **7** was the epimer of metabolite **6**. Therefore, the structure of metabolite **7** could be established as 10*R*,14-epoxy-7,11-dehydrocurcumol.

In conclusion, six curcumol metabolites were isolated from the biotransformation reactions with *C. blakesleana* (AS 3.970). The fungus demonstrated its ability to oxygenise the double bond between C-10 and C-14, and to form a new double bond between C-7 and C-11.



4

Figure 3. Key NOESY correlations of **4**.

3. Experimental

3.1 General experimental procedures

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 NMR, ^{13}C NMR, DEPT, ^{1}H—^{1}H-COSY, HMQC, 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, USA) with PDA996 (Waters, USA) 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.

3.2 Microorganisms

Cunninghamella blakesleana (AS 3.970), which were purchased from China General Microbiological Culture Collection Centre, was a gift from Dr. Dean Guo.

3.3 Substrate material

Curcumol **1** (99% as the pureness, detected with HPLC) was isolated from the essential oil of *Curcuma wenyujin*, and characterised by chemical and spectral methods as described in the literature [8]. The substrate was dissolved in acetone and diluted to 4.0 mg/ml before use.

3.4 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 1 h, then the extract was filtered and the filtrate were added with water to 1 L after addition of 20 g of glucose.

3.5 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 ml of the substrate solution was added into each flask with 2-day-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 days 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 visualised 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-day-old microorganism cultures, and after another 5 days of incubation, the culture media were collected, extracted and concentrated as described above.

3.6 Isolation and purification of the metabolites

The obtained residue (5.6 g) was separated on a silica gel (200–300 mesh) column (3.5 × 42 cm), 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. From fraction IX, 116.9 mg of metabolite **2** was isolated (5.8% yield) by recrystallisation in acetone. Fraction VI (1.2 g) was subjected to silica gel column (3.5 × 28 cm) chromatography, eluted with a mixture of cyclohexane/acetone (20:1, 10:1, 5:1, 700 ml for each gradient eluent) to yield 183.7 mg (9.2% yield) of metabolite **4** (950–1250 ml) and 32.3 mg (1.6% yield) of metabolite **5** (between 250 ml and 350 ml). The mother solution of metabolite **4** was further separated by preparative HPLC to yield 12.8 mg (0.6% yield) of **6** and 2.0 mg (0.1% yield) of metabolite **7** (Inertsil C_{18} Prepare column 20.0 × 250 mm, 55% MeOH/H_2O as mobile phase, flow rate: 8 ml/min). Fraction X (1.0 g) was subjected to silica gel column (3.0 × 40 cm) chromatography, eluted with cyclohexane/acetone (10:1, 5:1, 2:1, 700 ml for each gradient eluent) to yield 36.8 mg (1.8% yield) of metabolite **3** as crystals (between 1350 ml and 1700 ml).

3.6.1 14-Hydroxy-9*E* curcumol (2). Colourless crystals (Acetone). $[\alpha]_D^{25}$ 27.5 (*c* 0.48 MeOH); IR (KBr) ν_{max} 3434.6, 2958.3, 2345.0, 1655.6, 1457.9, 1297.9, 1155.1, 1020.2, 945.9, 690.4 cm^{-1}. ESI-MS *m/z*: 275 $[M + Na]^+$, 527 $[2M + Na]^+$, 235 $[M - OH]^+$.

Table 1. ^{13}C NMR data of curcumol (**1**) and its metabolites.

Carbon	*1*[a]	*2*[a]	*3*[b]	*4*[b]	*5*[c]	*6*[b]	*7*[b]
1	54.5	49.6	54.6	53.0	54.2	52.0	53.1
2	28.2	27.7	25.5	25.3	25.8	24.7	25.9
3	30.9	31.2	30.6	31.4	31.1	31.6	31.4
4	39.4	40.2	40.7	41.0	40.4	40.2	40.3
5	88.1	87.1	87.8	87.8	86.8	87.7	87.6
6	34.7	36.5	34.2	35.6	34.5	37.1	36.8
7	56.5	59.6	56.3	55.7	55.9	134.8	134.4
8	104.5	103.4	105.3	105.8	105.1	104.2	103.9
9	38.8	123.6	39.9	38.3	38.7	41.7	41.9
10	144.7	144.0	74.9	58.2	57.1	59.2	58.9
11	28.7	30.8	29.4	31.3	29.5	128.0	128.1
12	21.5	21.4	21.5	21.6	21.5	19.2	19.3
13	23.1	22.6	23.6	23.6	23.6	22.6	22.9
14	112.9	64.5	70.9	59.7	49.1	58.3	49.3
15	12.3	11.7	12.6	12.5	12.4	12.6	12.5

Spectra were recorded at 100 MHz NMR in $CDCl_3$ (a), CD_3OD (b) or $(CD_3)_2CO$ (c). Values are expressed in ppm downfield from TMS.

Table 2. ^{1}H NMR data of curcumol (**1**) and its metabolites.

^{1}H	1^{a}	2^{a}	3^{b}	4^{b}	5^{c}	6^{b}	7^{b}
1	2.18 (dd, 12, 9)	2.02 (br. t, 10)	1.64 (br. t, 11)	1.53 (dd, 11, 8)	1.26 (dd, 12, 9)	1.52 (m)	1.33 (dd, 11, 9)
2	1.65	1.57 (m)	1.51 (m)	1.58 (m)	1.65	1.64 (m)	1.72
	(2H, m)	1.90 (m)	1.73 (m)	1.73 (m)	(2H, m)	1.83 (m)	(2H, m)
3	1.47 (m)	1.59 (m)	1.30 (m)	1.43 (m)	1.36 (m)	1.50 (m)	1.48 (m)
	1.90 (m)	1.92 (m)	1.85 (m)	1.89 (m)	1.84 (m)	1.98 (m)	1.91 (m)
4	1.88 (m)	1.83 (m)	1.83 (m)	1.87 (m)	1.88 (m)	1.96 (m)	1.97 (m)
6	1.18 (dd, 12, 7)	1.25 (dd, 13, 7)	1.92 (dd, 12, 8)	1.36 (dd, 13, 7)	1.52 (dd, 13, 7)	2.20 (dt, 15, 1)	2.27 (dt, 15, 1)
	2.14 (dd, 12, 12)	2.21 (dd, 13, 11)	2.01 (dd, 12, 12)	2.21 (dd, 13, 12)	2.13 (dd, 13, 12)	2.59 (dt, 15, 2)	2.62 (dt, 15, 2)
7	1.47 (m)	1.65 (m)	1.53 (m)	1.63 (o)	1.62 (m)	–	–
9	2.55 (2H, br. s)	5.83 (1H, br. s)	1.52 (d, 14)	1.65 (br. d, 14)	1.49 (dd, 15, 1)	1.58 (br. d, 12)	1.48 (dd, 14, 1)
			1.90 (d, 14)	2.12 (br. d, 14)	2.27 (dd, 15, 1)	2.25 (dd, 12, 1)	2.39 (br. d, 14)
11	1.73 (m)	1.48 (m)	2.30 (m)	1.63 (o)	2.11 (m)	–	–
12	1.01 (3H, d, 6)	1.00 (3H, d, 6)	0.96 (3H, d, 5)	1.00 (3H, d, 6)	0.95 (3H, d, 6)	1.79 (3H, dd, 1, 2)	1.77 (3H, dd, 1, 2)
13	0.87 (3H, d, 6)	0.88 (3H, d, 7)	0.89 (3H, d, 7)	0.94 (3H, d, 6)	0.91 (3H, d, 6)	1.67 (3H, br. s)	1.68 (3H, br. s)
14	4.88 (2H, s)	4.09 (2H, br. s)	3.27 (d, 11)	2.76 (2H, s)	2.46 (d, 5)	2.50 (d, 5)	2.46 (d, 4)
			3.37 (d, 11)		2.51 (d, 5)	2.62 (dd, 5, 2)	2.53 (d, 4)
15	1.00 (3H, d, 6)	1.02 (3H, d, 6)	0.94 (3H, d, 5)	0.98 (3H, d, 6)	0.94 (3H, d, 6)	0.99 (3H, d, 6)	0.99 (3H, d, 6)

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

HRFAB-MS *m/z* 253.1796 $[M + H]^+$ (calcd for $C_{15}H_{25}O_3$, 253.1798). ^1H NMR and ^{13}C NMR data are listed in tables 1 and 2, respectively.

3.6.2 10*R*,14-Dihydroxy curcumol (3). Colourless crystals (Acetone). $[\alpha]_D^{25}$ 23.7 (*c* 0.68 MeOH); IR (KBr) ν_{max} 3569.6, 3303.5, 2955.4, 2362.4, 1458.9, 1367.3, 1110.8, 1004.7, 943.0, 670.1 cm^{-1}. ESI-MS *m/z*: 293 $[M + Na]^+$, 563 $[2M + Na]^+$, 275 $[M + Na—H_2O]^+$. HRFAB-MS *m/z* 271.1904 $[M + H]^+$ (calcd for $C_{15}H_{27}O_4$, 271.1904). ^1H NMR and ^{13}C NMR data are listed in tables 1 and 2, respectively.

3.6.3 10*S*,14-Epoxy curcumol (4). Colourless crystals (MeOH). $[\alpha]_D^{25}$ 51.3 (*c* 0.54 MeOH); IR (KBr) ν_{max} 3415.3, 2953.4, 1460.8, 1335.5, 1132.9, 1036.5, 998.9, 932.4, 827.3 cm^{-1}. ESI-MS *m/z*: 275 $[M + Na]^+$, 527 $[2M + Na]^+$, 256 $[M + Na—H_2O]^+$. HRFAB-MS *m/z* 253.1798 $[M + H]^+$ (calcd for $C_{15}H_{25}O_3$, 253.1798). ^1H NMR and ^{13}C NMR are listed in tables 1 and 2, respectively.

3.6.4 10*R*,14-Epoxy curcumol (5). Colourless crystals (MeOH). $[\alpha]_D^{25}$ − 38.2 (*c* 0.54 MeOH); IR (KBr) ν_{max} 3449.1, 2956.3, 2366.2, 1458.9, 1340.3, 1224.6, 1096.3, 926.6, 754.0 cm^{-1}. ESI-MS *m/z*: 275 $[M + Na]^+$, 256 $[M + Na—H_2O]^+$, 235 $[M - HO]^+$. HRFAB-MS *m/z* 253.1797 $[M + H]^+$ (calcd for $C_{15}H_{25}O_3$, 253.1798). ^1H NMR and ^{13}C NMR data are listed in tables 1 and 2, respectively.

3.6.5 10*S*,14-Epoxy-7,11-dehydrocurcumol (6). Colourless crystals (MeOH). $[\alpha]_D^{25}$ 46.9 (*c* 0.49 MeOH); IR (KBr) ν_{max} 3403.7, 2933.2, 2361.4, 1691.3, 1456.9, 1325.8, 1170.6, 1036.5, 986.4, 879.4 cm^{-1}. ESI-MS *m/z*: 273 $[M + Na]^+$, 523 $[2M + Na]^+$, 231 $[M - H_2O]^+$. HRFAB-MS *m/z* 251.1641 $[M + H]^+$ (calcd for $C_{15}H_{23}O_3$, 251.1642). ^1H NMR and ^{13}C NMR are listed in tables 1 and 2, respectively.

3.6.6 10*R*,14-Epoxy-7,11-dehydrocurcumol (7). Colourless crystals (MeOH). $[\alpha]_D^{25}$ − 32.4 (c 0.28 MeOH); IR (KBr) ν_{max} 3427.3, 2936.5, 2365.7, 1688.3, 1404.9, 1330.8, 1106.9, 955.6, 850.4, 614.2 cm^{-1}. ESI-MS *m/z*: 273 $[M + Na]^+$, 523 $[2M + Na]^+$, 255 $[M + Na—H_2O]^+$. HRFAB-MS *m/z* 251.1641 $[M + H]^+$ (calcd for $C_{15}H_{23}O_3$, 251.1642). ^1H NMR and ^{13}C NMR are listed in tables 1 and 2, respectively.

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