New Derivatives of Nonactic and Homononactic Acids from *Bacillus pumilus* Derived from *Breynia fruticosa*

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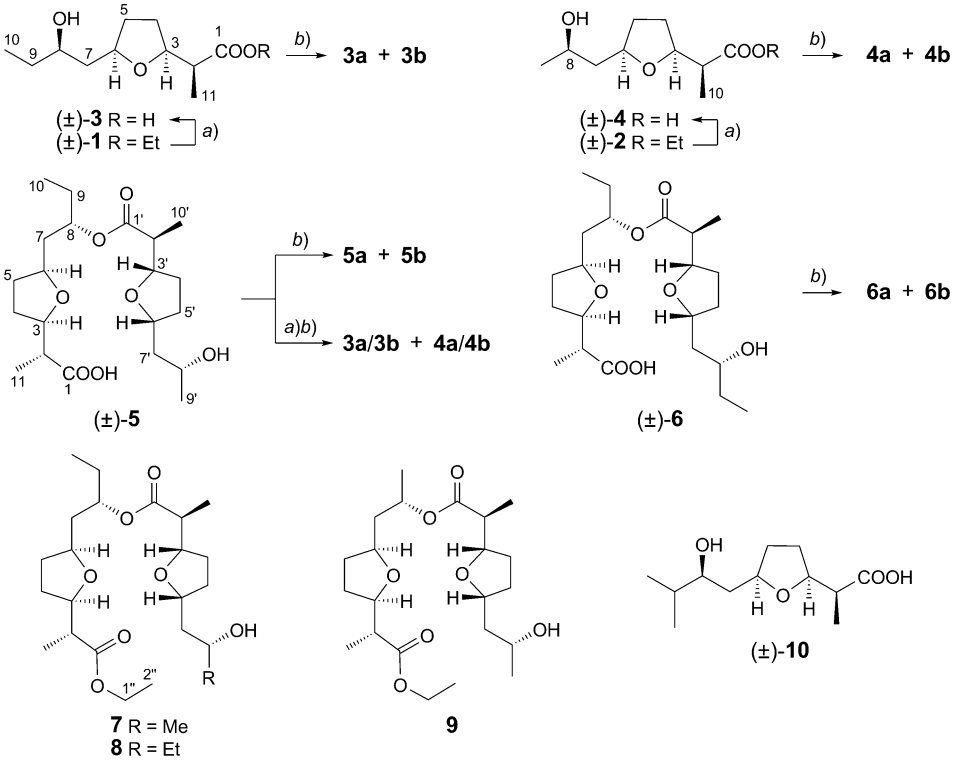
Six new nonactic and homononactic acid derivatives, ethyl homononactate (**1**), ethyl nonactate (**2**), homononactyl homononactate (**6**), ethyl homononactyl nonactate (**7**), ethyl homononactyl homononactate (**8**), and ethyl nonactyl nonactate (**9**), as well as four known compounds, homononactic acid (**3**), nonactic acid (**4**), homononactyl nonactate (**5**), and bishomononactic acid (**10**), were isolated from culture broth of *Bacillus pumilus* derived from *Breynia fruticosa*. The structures of new compounds were elucidated by spectroscopic analysis and chemical methods. The optical purities of **1**–**6** were determined by HPLC/MS after treatment with L-phenylalanine methyl ester. The dimeric compounds **5**–**9** showed weak cytotoxic activities against five human cancer cell lines (IC_{50} 19–100 μg/ml).

Introduction. – Endophytes from medicinal plants have been considered as a major source for bioactive natural products for a long time [1]. Xishuangbanna, Yunnan, P. R. China, possesses abundant medicinal plant resources because of its unique geographical and climatic characteristics. In a previous work, the co-authors at Yunnan University had studied the diversity of microorganisms from medicinal plants collected from Xishuangbanna, and more than 2,000 strains were isolated [2]. As a subsequent work, searching for new bioactive metabolites from microbial sources associated with Xishuangbanna medicinal plants, 87 strains were fermented in small amounts (100 ml) with four culture broths, respectively, and the metabolites were assessed by the chemical screening method. *Bacillus pumilus* (YIM 56368) was selected for investigations based on HTLC and HPLC analyses.

Bacillus pumilus derived from *Breynia fruticosa* (Euphorbiaceae), which is a Dai medicinal plant, distributed in Xishuangbanna, used for treating dermatitis, eczema, sore, and furuncle in local medicine. In this article, we describe the isolation and structure elucidation of six new nonactic and homononactic acid derivatives from the fermentation broth of *B. pumilus*, named ethyl homononactate (**1**), ethyl nonactate (**2**), homononactyl homononactate (**6**), ethyl homononactyl nonactate (**7**), ethyl homononactyl homononactate (**8**), and ethyl nonactyl nonactate (**9**), along with the known compounds homononactic acid (**3**) [3][4], nonactic acid (**4**) [3][4], homononactyl

Scheme



a) 0.1N KOH, 95% EtOH, r.t. *b*) L-Phenylalanine methyl ester, *N*,*N*′-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), CH_2Cl_2, r.t.

nonactate (**5**) [5], and bishomononactic acid (**10**) [6] (*Scheme*). The structures of the new compounds were determined on the basis of spectroscopic chemical methods.

Nonactic and homononactic acids were the building units of macrotetrolide antibiotics, such as nonactin, monactin, dinactin, trinactin, *etc.* [4][7]. Although total synthesis of the derivatives of homonoactic and nonactic acids had been accomplished previously, the optical-rotation values of synthetic pure chiral molecules were very small, just between 9–14° [6][8–10]. Thus, it was very difficult to confirm whether the natural derivatives of them existed in optically pure or racemic form only on the basis of optical-rotation values [3][11]. Furthermore, there was no report in the literature to verify homonoactic and nonactic acid derivatives isolated from natural sources, as a mixture of enantiomers. In the present work, ethyl homononactate (**1**), ethyl nonactate (**2**), homononactic acid (**3**), nonactic acid (**4**), homononactyl nonactate (**5**), and homononactyl homononactate (**6**) were confirmed as partially racemic mixtures by treatment with L-phenylalanine methyl ester, and the derivatives **3a**/**3b**, **4a**/**4b**, **5a**/**5b**, and **6a**/**6b** (*Scheme*, *Table 1*) were analyzed by HPLC/MS. Furthermore, biological assays for the cytotoxic activities of these isolated compounds were performed.

Table 1. *Analysis of* L-*Phenylalanine Amide Derivatives of* **3**–**6** *Obtained from the Esters* **1**, **2**, *and* **5**

Compound	Derivatives	t_R [min]	Peak area [%]
1	**3a**/**3b**	4.974/6.098	14 : 100
2	**4a**/**4b**	4.626/5.792	100 : 71
3	**3a**/**3b**	4.974/6.098	79 : 100
4	**4a**/**4b**	4.626/5.792	100 : 34
5	**5a**/**5b**	3.949/4.679	57 : 100
	3a/**3b**, **4a**/**4b**	4.974/6.098, 4.626/5.792	48 : 100, 100 : 62
6	**6a**/**6b**	6.345/6.959	100 : 51

Results and Discussion. – Compound **1** was obtained as colorless oil, and its molecular formula was deduced as $C_{13}H_{24}O_4$ from HR-ESI-MS (*m*/*z* 267.1562 ($[M+Na]^+$, $C_{13}H_{24}NaO_4^+$; calc. 267.1572)). The IR spectrum revealed the presence of OH (3329 cm^{-1}) and C=O groups (1739 cm^{-1}). The ^{1}H-NMR spectrum (*Table 2*) exhibited one O-bearing CH_2 signal (δ(H) 4.10–4.19 (*m*), three CH–O signals (δ(H) 4.10–4.16 (*m*), 3.98 (*q*-like, $J=6.6$), 3.71–3.75 (*m*)), and one CH (δ(H) 2.51 (*quint.*-like, $J=7.2$), four CH_2, and three Me signals. The ^{13}C-NMR spectrum of **1** showed all 13 signals indicated by the molecular formula, including that of one ester C=O group (δ(C) 174.8). The HSQC spectrum allowed the assignment of all the H-atoms to the corresponding C-atoms. The close similarity of NMR data indicated compound **1** to be structurally related to homononactic acid (**3**). An additional EtO signal suggested **1** was ethyl ester derivative of **3**, which was confirmed by HMBC between the signal at δ(H) 4.10–4.19 (*m*) and that at δ(C) 174.8. Thus, the structure of **1** was elucidated as ethyl homononactate. Homononactic acid (**3**), the precursor of **1**, was also isolated from the same strain. The absolute configuration of **3** ($[\alpha]_D^{20}=-6.38$ ($c=3.6$, MeOH)) was inferred first by chemical derivatization and optical-purity analysis. Compound **3** was treated with L-phenylalanine methyl ester to yield two derivatives, **3a** and **3b**, with a peak area ratio of 79 : 100 determined by HPLC/MS. Compounds **3a** and **3b** were isolated by preparative HPLC, and their structures were confirmed by ^{1}H- and ^{13}C-NMR, and mass spectra. Therefore, **3** was determined as a partially racemic mixture composed of (+)- and (−)-homononactic acid. By the same method, **1** ($[\alpha]_D^{20}=+28.9$ ($c=0.95$, MeOH)) was reacted with L-phenylalanine methyl ester after alkaline hydrolysis, and two compounds, **3a** and **3b**, were detected by HPLC/MS with a peak integral ratio of 14 : 100. Thus, the structure of **1** was determined as a mixture composed of (+)- and (−)-ethyl homononactate.

Compound **2** was isolated as colorless oil, and its molecular formula was deduced as $C_{12}H_{22}O_4$ from the HR-ESI-MS (*m*/*z* 253.1418 ($[M+Na]^+$, $C_{12}H_{22}NaO_4^+$; calc. 253.1410)). The ^{1}H- and ^{13}C-NMR data indicated a close resemblance of **2** to the known compound nonactic acid (**4**; *Table 2*). The only difference was the presence of an additional EtO group in **2**. The location of the EtO group at C(1) was confirmed by HMBC between the signals for CH_2O (δ(H) 4.11–4.18 (*m*) and the C=O C-atom (δ(C) 174.8). To determine the absolute configurations of **2** and **4** ($[\alpha]_D^{20}=6.25$ ($c=1.6$, MeOH)), their L-phenylalanine methyl ester derivatives were prepared and analyzed by HPLC/MS. Two products **4a** and **4b** were detected respectively. Therefore, the structure of **4** was established as a pair of enantiomers of (+)- and (−)-nonactic acid

Table 2. *^{1}H- and ^{13}C-NMR Data of* **1** *and* **2** *in $CDCl_3$.* δ in ppm, J in Hz.

Position	**1**[a])		**2**[b])	
	δ(H)	δ(C)	δ(H)	δ(C)
1		174.8		174.8
2	2.51 (*quint.*-like, $J=7.2$)	45.3	2.52 (*quint.*-like, $J=7.2$)	45.3
3	3.98 (*q*-like, $J=6.6$)	81.0	3.98 (*q*-like, $J=6.6$)	81.1
4	1.97–2.01, 1.59–1.65 (2*m*)	28.7	1.98–2.03, 1.61–1.65 (2*m*)	28.7
5	1.97–2.01, 1.59–1.65 (2*m*)	30.6	1.97–2.02, 1.61–1.65 (2*m*)	30.5
6	4.10–4.16 (*m*)	76.6	4.11–4.18 (*m*)	77.2
7	1.72 (*ddd*, $J=14.4$, 7.8, 4.2), 1.66 (*ddd*, $J=14.4$, 7.2, 3.0)	40.6	1.74 (*ddd*, $J=13.3$, 8.0, 4.6), 1.62–1.68 (*m*)	42.6
8	3.71–3.75 (*m*)	70.4	3.96–4.06 (*m*)	65.2
9	1.50–1.54, 1.44–1.48 (2*m*)	29.9	1.21 (*d*, $J=6.3$)	23.1
10	0.93 (*t*-like, $J=7.2$)	10.1	1.13 (*d*, $J=6.6$)	13.5
11	1.11 (*d*, $J=7.2$)	13.4		
1′	4.10–4.19 (*m*)	60.4	4.11–4.18 (*m*)	60.5
2′	1.25 (*t*, $J=7.2$)	14.1	1.27 (*t*, $J=7.0$)	14.2
OH	2.78 (br. *s*)		2.85 (br. *s*)	

[a]) At 600 and 150 MHz for ^{1}H- and ^{13}C-NMR, respectively. [b]) At 300 and 75 MHz for ^{1}H- and ^{13}C-NMR, respectively.

with predominance of (+)-nonactic acid. Compound **2** was also a mixture of (+)- and (−)-ethyl nonactate with predominance of (+)-enantiomers.

The ^{1}H- and ^{13}C-NMR, and ESI-MS data of **5** were identical to those of the known compound homononactyl nonactate which had been obtained from *Streptomyces globisporus* as reported in [5]. Unfortunately, the authors had failed to determine the relative and absolute configuration of homononactyl nonactate by alkaline hydrolysis. The structure of related compound feigrisolide C was corrected later, and the absolute configuration was identified as (2′*S*,3′*S*,6′*R*,8′*R*)-homononactoyl (2*R*,3*R*,6*S*,8*S*)-nonactic acid by means of total synthesis [12][13]. By careful comparison of the ^{13}C-NMR data with those in [13], we could deduce the structure of **5** either as (2*R*,3*R*,6*S*,8*S*)-homononactyl (2′*S*,3′*S*,6′*R*,8′*R*)-nonactate, or as an enantiomer mixture. A chemical derivatization of **5** with L-phenylalanine methyl ester was conducted, and two products **5a** and **5b** were detected in a ratio of 57 : 100. Meanwhile, **5** was hydrolyzed and treated with L-phenylalanine methyl ester. Four peaks for **3a**/**3b** and **4a**/**4b** were detected with ratios of 48 : 100 and 100 : 62, respectively, by HPLC/MS. Thus, compound **5** was determined as a partially racemic mixture of (2*R*,3*R*,6*S*,8*S*)-homononactyl (2′*S*,3′*S*,6′*R*,8′*R*)-nonactate.

Compound **6** was isolated as colorless oil, and its molecular formula, $C_{22}H_{38}O_7$, was deduced from HR-ESI-MS (*m*/*z* 413.2537 ($[M-H]^-$, $C_{22}H_{37}O_7^-$; calc. 413.2539)). The IR spectrum indicated the presence of OH (3422 cm^{-1}) and C=O (1729 cm^{-1}) functionalities. The ^{13}C-NMR spectrum of **6** exhibited 22 C-atom signals, which were assigned to four Me, eight CH_2, and eight CH groups, and two quaternary C-atoms (*Table 3*). The ^{1}H- and ^{13}C-NMR data of **6** suggested that there were two homononactic acid residues in **6**, a HMBC between the signal at δ(H) 4.96 (*quint.*-like, $J=6.0$) and that at δ(C) 174.6 confirmed the structure as homononactyl homononactate. The ^{1}H-

and ^{13}C-NMR, and MS data of **6** were identical to feigrisolide D reported in [12], which was isolated from the culture broth of *Streptomyces griseus*, as well as feigrisolides A–C, and the structure of feigrisolide D was proposed to be a macrodiolide. Feigrisolides A–C were corrected to nonactic acid, homononactic acid, and homononactyl nonactate, respecitvely, by synthesis [13–15]. Although, no synthesis was reported verifying the incorrect structure of feigrisolide D, we still could confirm that feigrisolide D possessed the same structure as **6** according to identical spectral data. The same derivatization method as in the case of **5** was applied to **6**, and two compounds **6a** and **6b** with a ratio of 100:51 were identified by HPLC/MS. So, **6** was elucidated as a racemic mixture composed of (2*R*,3*R*,6*S*,8*S*)-homononactyl (2′*S*,3′*S*,6′*R*,8′*R*)-homononactate and *ent*-**6**.

Table 3. *^{13}C-NMR Data of* **5**–**9**. δ in ppm.

Position	**5**[a])[c])	**6**[b])[c])	**7**[b])[c])	**8**[b])[c])	**9**[b])[d])
1	178.6	177.2	174.9	174.8	174.9
2	46.7	45.1	45.4	45.4	45.4
3	81.9	80.5	80.3	80.3	80.4
4	29.4	29.0	28.4	28.4	28.4
5	32.4	31.1	31.4	31.4	31.4
6	77.7	76.6	76.5	76.5	76.6
7	41.5	40.2	40.2	40.2	42.5
8	74.9	73.2	73.7	73.8	69.5
9	28.6	27.3	27.4	27.4	20.5
10	9.7	9.4	9.3	9.3	13.2
11	13.8	13.4	13.1	13.5	
1′	176.5	174.6	174.3	174.4	174.2
2′	47.5	45.5	45.5	45.5	45.4
3′	82.0	80.9	80.8	80.9	80.9
4′	29.7	28.8	28.6	28.6	28.6
5′	32.2	30.5	30.6	30.6	30.6
6′	77.9	77.0	76.9	76.9	77.2
7′	46.3	40.6	43.0	40.8	42.9
8′	66.1	70.3	65.1	70.3	65.1
9′	24.3	29.9	23.3	30.0	23.3
10′	14.2	10.1	13.5	10.1	13.2
11′		13.8		13.5	
1″			60.3	60.3	60.3
2″			14.2	14.2	14.2

[a]) In CD_3OD. [b]) In $CDCl_3$. [c]) At 75 MHz. [d]) At 150 MHz.

Compound **7** was isolated as colorless oil, and its molecular formula, $C_{23}H_{40}O_7$, was determined by HR-ESI-MS (*m*/*z* 451.2680 ($[M+Na]^+$, $C_{23}H_{40}NaO_7^+$; calc. 451.2672)). The ^{1}H- and ^{13}C-NMR spectra were very similar to those of **5**, the spectra of **7** indicating an additional EtO group (δ(H) 4.12–4.18 (*m*, 2 H), 1.26 (*t*, *J* = 7.2, 3 H); δ(C) 60.3, 14.2). The HMBC experiment confirmed that **7** was the ethyl ester of **5**. Complete assignments of the ^{1}H- and ^{13}C-NMR spectra of **7** were achieved (*Tables 3* and *4*) with the aid of HSQC, HMBC, and NOESY experiments. Because of the lack of material,

Table 4. *^{1}H-NMR Data of* **5**–**9**. δ in ppm, J in Hz.

Position	**5**[a])[c])	**6**[b])[c])	**7**[b])[c])	**8**[b])[c])	**9**[b])[d])
2	2.40–2.45 (*m*)	2.49 (*quint.*-like, J=7.2)	2.50 (*quint.*-like, J=7.2)	2.45–2.55 (*m*)	2.49 (*quint.*-like, J=7.2)
3	3.94–3.96 (*m*)	3.95–4.01 (*m*)	3.96–4.03 (*m*)	3.98–4.03 (*m*)	3.97–4.02 (*m*)
4	1.91–1.94 (*m*),	1.97–2.03 (*m*),	1.95–2.02 (*m*),	1.90–1.96 (*m*),	1.92–1.96 (*m*),
	1.64–1.67 (*m*)	1.66–1.70 (*m*)	1.59–1.63 (*m*)	1.63–1.68 (*m*)	1.60–1.64 (*m*)
5	1.97–2.00 (*m*),	2.01–2.05 (*m*),	1.95–2.02 (*m*),	1.97–2.02 (*m*),	1.96–2.02 (*m*),
	1.46–1.54 (*m*)	1.56–1.58 (*m*)	1.48–1.53 (*m*)	1.50–1.55 (*m*)	1.50–1.55 (*m*)
6	3.84–3.90 (*m*)	3.95–4.01 (*m*)	3.88 (*quint.*-like, J=6.3)	3.85–3.91 (*m*)	3.88 (*quint.*-like, J=6.0)
7	1.71 (*t*-like, J=6.3)	1.74–1.78 (*m*)	1.72–1.78 (*m*)	1.77–1.83 (*m*),	1.79 (*ddd*, J=13.2, 7.2, 5.4),
				1.72–1.78 (*m*)	1.69–1.74 (*m*)
8	3.87–4.93 (*m*)	4.96 (*quint.*-like, J=6.0)	4.93 (*quint.*-like, J=6.3)	4.94 (*quint.*-like, J=6.6)	5.00 (*sext.*-like, J=6.6)
9	1.61–1.64 (*m*),	1.58–1.64 (*m*),	1.62–1.68 (*m*),	1.63–1.68 (*m*),	1.23 (*d*, J=6.0)
	1.51–1.56 (*m*)	1.55–1.57 (*m*)	1.54–1.59 (*m*)	1.54–1.60 (*m*)	
10	0.86 (*t*, J=7.5)	0.88 (*t*, J=7.2)	0.88 (*t*, J=7.2)	0.89 (*t*, J=7.2)	1.09 (*d*, J=7.2)
11	1.06 (*d*, J=6.7)	1.15 (*d*, J=7.29	1.09 (*d*, J=6.6)	1.11 (*d*, J=6.3)	
2′	2.38–2.42 (*m*)	2.49 (*quint.*-like, J=7.2)	2.51 (*quint.*-like, J=7.2)	2.45–2.55 (*m*)	2.49 (*quint.*-like, J=7.2)
3′	3.90–3.94 (*m*)	3.97–4.03 (*m*)	3.96–4.03 (*m*)	3.98–4.03 (*m*)	3.97–4.02 (*m*)
4′	1.94–1.97 (*m*),	1.97–2.03 (*m*),	1.90–1.96 (*m*),	1.97–2.02 (*m*),	1.95–2.02 (*m*),
	1.58–1.61 (*m*)	1.58–1.60 (*m*)	1.62–1.68 (*m*)	1.59–1.62 (*m*)	1.60–1.64 (*m*)
5′	1.94–1.97 (*m*),	1.97–2.03 (*m*),	1.95–2.02 (*m*),	1.97–2.02 (*m*),	1.96–2.00 (*m*),
	1.46–1.54 (*m*)	1.59–1.61 (*m*)	1.58–1.62 (*m*)	1.59–1.62 (*m*)	1.59–1.62 (*m*)
6′	3.96–4.00 (*m*)	4.13–4.19 (*m*)	4.09–4.16 (*m*)	4.10–4.17 (*m*)	4.10–4.13 (*m*)
7′	1.52–1.57 (*m*)	1.67–1.71 (*m*)	1.68–1.74 (*m*),	1.70–1.75 (*m*),	1.69–1.74 (*m*),
			1.62–1.68 (*m*)	1.65–1.70 (*m*)	1.63 (*ddd*, J=14.4, 7.2, 3.0)
8′	3.81–3.86 (*m*)	3.71–3.77 (*m*)	3.98–4.06 (*m*)	3.70–3.76 (*m*)	4.00–4.06 (*m*)
9′	1.13 (*d*, J=6.3)	1.50–155 (*m*),	1.19 (*d*, J=6.3)	1.50–1.55 (*m*),	1.19 (*d*, J=6.0)
		1.45–1.50 (*m*)		1.44–1.50 (*m*)	
10′	1.06 (*d*, J=6.7)	0.93 (*t*, J=7.2)	1.11 (*d*, J=6.9)	0.93 (*t*, J=7.2)	1.10 (*d*, J=7.2)
11′		1.12 (*d*, J=7.2)		1.15 (*d*, J=6.6)	
1″			4.12–4.18 (*m*)	4.10–4.17 (*m*)	4.11–4.19 (*m*)
2″			1.26 (*t*, J=7.2)	1.27 (*t*, J=7.2)	1.26 (*t*, J=7.2)

[a]) In CD_3OD. [b]) In $CDCl_3$. [c]) At 300 MHz. [d]) At 600 MHz.

we could not elucidate the optical purity of **7** by hydrolysis or derivatization. However, based on comparison of the optical rotation value of **7** ($[\alpha]_D^{20} = -12.53$ ($c = 0.76$, MeOH)) with that of **5** ($[\alpha]_D^{20} = -7.63$ ($c = 3.10$, MeOH)) of the same biogenetic source, we could deduce that, like **5**, **7** was a partial racemic mixture consisting of ethyl (2*R*,3*R*,6*S*,8*S*)-homononactyl (2′*S*,3′*S*,6′*R*,8′*R*)-nonactate and ethyl (2*S*,3*S*,6*R*,8*R*)-homononactyl (2′*R*,3′*R*,6′*S*,8′*S*)-nonactate.

Compound **8** had the molecular formula $C_{24}H_{42}O_7$, as deduced from HR-ESI-MS. Analysis of ^{1}H- and ^{13}C-NMR data indicated a close structural relationship with **6**, except for another EtO group (δ(H) 4.10–4.17 (*m*, 2 H), 1.27 (*t*, *J* = 7.2, 3 H); and δ(C) 60.3, 14.2) in **8**. HMBC from CH_2(1″) (δ(H) 4.10–4.17) and Me(2″) (δ(H) 1.27) to C(1′) (δ(C) 174.4) evidenced the gross structure of **8** as an ethyl ester of **6**. Both **6** and **8** were optically inactive; accordingly, **8** was also a racemic compound composed of ethyl (2*R*,3*R*,6*S*,8*S*)-homononactyl (2′*S*,3′*S*,6′*R*,8′*R*)-homononactate and *ent*-**8**.

Compound **9** had the molecular formula $C_{22}H_{38}O_7$ determined by HR-ESI-MS. The ^{1}H- and ^{13}C-NMR spectra were very similar to those of known dimeric nonactic acid [16], with the exception of one more EtO group (δ(H) 4.11–4.19 (*m*, 2 H); 1.26 (*t*, *J* = 7.2, 3 H); δ(C) 60.3, 14.2) in **9**. All the spectral data suggested that **9** was the ethyl ester of dimeric nonactic acid, which was confirmed by HMBCs. Complete assignments of the ^{1}H- and ^{13}C-NMR spectra of **9** were achieved (*Tables 3* and *4*) with the aid of HSQC, HMBC, and NOESY experiments. Hydrolysis or derivatization was not conducted on **9** because of lack of material. By careful comparison of the ^{13}C-NMR data with those of feigrisolide C, which was obtained by total synthesis [15], all dimeric compounds **5**–**9** exhibited the same configuration as feigrisolide C. All of them consist of two parts of opposing optical rotations, *i.e.*, (−)-homononactyl (+)-nonactate (**5**), (−)-homononactyl (+)-homononactate (**6**), ethyl (−)-homononactyl (+)-nonactate (**7**), ethyl (−)-homononactyl (+)-homononactate (**8**), ethyl (−)-nonactyl (+)-nonactate (**9**), and their enantiomers. The structure of **9**, either an optically pure compound or a racemic mixture, could not be determined since only the optical-rotation value ($[\alpha]_D^{20} = 0$ ($c = 0.15$, MeOH)) was available.

Compounds **1**–**10** were evaluated for their cytotoxic activities against five human cancer cell lines, including human gastric carcinoma cell line (BGC-823), human hepatocellular liver carcinoma cell line (HepG2), human large-cell lung carcinoma cell line (H460), human cervix carcinoma cell line (HeLa), and human colon carcinoma cell line (HCT116) with MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) method [17]. Dimeric compounds **5**–**9** showed weak cytotoxic activities (*Table 5*), whereas monomers **1**–**4** and **10** were inactive against the five cell lines ($IC_{50} > 100$ μg/ml).

Conclusions. – Six new derivatives of homonoactic and nonactic acids were isolated from the culture broth of *B. fruticosa* and identified. The enantiomeric excesses of ethyl homononactate (**1**), ethyl nonactate (**2**), homononactic acid (**3**), nonactic acid (**4**), homononactyl nonactate (**5**), and homononactyl homononactate (**6**) were revealed by chemical derivatization and HPLC/MS analysis. Dimeric compounds **5**–**9** showed weak cytotoxic activities compared to the monomer compounds **1**–**4** and **10**.

Table 5. *Cytotoxic* (IC_{50} [μg/ml]) *Activities of Compounds* **5**–**9**

Compound	H460	HeLa	HCT116	BGC-823	HepG-2
5	19.1	27.8	29.8	32.2	58.4
6	61.6	87.5	73.2	>100	67.9
7	43.2	65.8	71.4	>100	>100
8	78.8	97.7	>100	>100	>100
9	69.6	>100	>100	>100	>100
Adriamycin	0.57	0.56	0.80	0.86	0.29

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Experimental Part

General. Agilent 1290-6420 Triple Quadrupole LC/MS system with a *Agilent SB-C18* column (2.1 × 50 mm, 1.8 μm) was used to analyze the L-phenylalanine methyl ester derivatives. Column chromatography (CC): silica gel (SiO_2; 100–200 mesh and 200–300 mesh; *Qingdao Marine Chemical Ltd.*, Qingdao, P. R. China), *Sephadex LH-20* (*GE Healthcare Bio-scienses AB*, SE-Uppsala), and *YMC*GEL ODS-A* (S-50 μm, 12 nm; *YMC Co., Ltd.*, Kyoto, Japan). HPLC: *Waters 600-2487* chromatograph with a *YMC pack ODS-A* column (30 × 250 mm, S-15 μm, 12 nm; flow rate, 10 ml/min), with MeOH/H_2O 70 : 30. Optical rotations: *SGW-1* automatic polarimeter (*Shanghai Precision & Scientific Instrument Co., Ltd.*, Shanghai, P. R. China). IR Spectra: *Nicolet Avatar 330* FT-IR spectrometer (KBr); $\tilde{\nu}$ in cm^{-1}. 1D- and 2D-NMR spectra: *Bruker ARX-300* or *Bruker AV-600* spectrometers; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. ESI-MS: *Thermo Finnigan LCQ* mass spectrometer; in *m*/*z*. HR-ESI-MS: *Waters LCT Premier XE* TOF mass spectrometer; in *m*/*z*. MTT assay was conducted on a microplate reader (*KHB ST-360*, *SH Kehua Laboratory System Co., Ltd.*, Shanghai, P. R. China).

Microbiological Material. The producing organism was isolated from a plant sample (*Breynia fruticosa*) collected from Xishuangbanna, Yunnan Province, P. R. China, in October 2009. The strain was identified as *Bacillus pumilus* by *Y. J.* based on morphological characteristics and 16S rRNA gene sequences. The strain (No. YIM 56368) was deposited with the Yunnan Institute of Microbiology, Yunnan University, P. R. China.

Fermentation, Extraction, and Isolation. A slant culture of the strain was inoculated into 500-ml *Erlenmeyer* flasks containing 100 ml of seed medium composed of yeast extract (4 $g\,l^{-1}$), glucose (4 $g\,l^{-1}$), malt extract (5 $g\,l^{-1}$), multiple vitamins soln. (1.0 ml), and trace-element soln. (1.0 ml), at pH 7.2, with no adjustment and cultured for 2 d at 28° on a rotary shaker at 180 rpm. This seed culture was used to inoculate the fermetation medium with 10% volume. The fermentation was carried out in a 500-ml *Erlenmeyer* flask containing 100 ml of fermentation medium containing soybean meal (10 $g\,l^{-1}$), peptone (2 $g\,l^{-1}$), glucose (20 $g\,l^{-1}$), soluble starch (5 $g\,l^{-1}$), yeast extract (2 $g\,l^{-1}$), NaCl (4 $g\,l^{-1}$), K_2HPO_4 (0.5 $g\,l^{-1}$), $MgSO_4 \cdot 7\,H_2O$ (0.5 $g\,l^{-1}$), $CaCO_3$ (2 $g\,l^{-1}$), at pH 7.8, with no adjustment, and incubated for 7 d at 28° on a rotary shaker at 180 rpm.

The completed fermentation broth (80 l) was separated into filtrate and mycelium by centrifugation. The culture filtrate was condensed to 10 l, and then partitioned in H_2O/AcOEt to yield 32.0 g of a dried AcOEt extract and an aq. residue. The AcOEt extract was subjected to CC (SiO_2; CH_2Cl_2/MeOH 50 : 1–1 : 1). The eluents were combined to nine fractions, *Frs. 1–9*, on the basis of TLC analysis. *Fr. 4* (3.48 g) was subjected to CC (*Sephadex LH-20*; MeOH) to give ten fractions, *Frs. 4.1–4.10*. *Fr. 4.3* was submitted to CC (*Sephadex LH-20*; MeOH) and further separated by CC (*ODS*; MeOH/H_2O 70 : 30) to yield **1** (23.0 mg), **2** (13.0 mg), and **9** (5.2 mg). *Fr. 5* (0.94 g) was purified by CC (*Sephadex LH-20*; MeOH) to give six fractions, *Frs. 5.1–5.6*. *Fr. 5.2* was separated by CC (*ODS*; MeOH/H_2O 70 : 30) to yield **7** (5.5 mg) and **8** (6.5 mg). *Fr. 6* (2.40 g) was subjected to by CC (*Sephadex LH-20*; MeOH) to afford 16 fractions,

Frs. 6.1–6.16. *Fr. 6.4* was purified by CC (*Sephadex LH-20*; MeOH) to afford five subfractions, *Frs. 6.4.1–6.4.5*. *Fr. 6.4.1* was separated by CC (*ODS*; MeOH/H_2O 80:20), and then further purified by CC (SiO_2; petroleum ether/AcOEt 5:3) to furnish **5** (46.0 mg) and **6** (22.0 mg). *Fr. 6.4.3* was separated by CC (*ODS*; MeOH/H_2O 40:60) to give **3** (350 mg) and **10** (9.5 mg). *Fr. 7* (1.03 g) was submitted to CC (*Sephadex LH-20*, MeOH) to afford eleven fractions, *Frs. 7.1–7.11*. *Fr. 7.4* was subjected to CC (*ODS*; MeOH/H_2O 40:60), and then further purified by CC (SiO_2; CH_2Cl_2/MeOH 40:1) to yield **4** (80 mg).

Ethyl Homononactate (= *Ethyl (2*S*)-2-{(2*S*,5*R*)-Tetrahydro-5-[(2*R*)-2-hydroxybutyl]furan-2-yl}propanoate*; **1**): Colorless oil. $[\alpha]_D^{20} = +28.9$ ($c = 0.95$, MeOH). IR (KBr): 3329, 2926, 2854, 1739, 1454, 1260, 1093, 1029. ^{1}H- and ^{13}C-NMR: see *Table 2*. ESI-MS: 267 ($[M+Na]^+$), 511 ($[2M+Na]^+$), 245 ($[M+H]^+$). HR-ESI-MS: 267.1562 ($[M+Na]^+$, $C_{13}H_{24}NaO_4^+$; calc. 267.1572).

Ethyl Nonactate (= *Ethyl (2*S*)-2-{(2*S*,5*R*)-Tetrahydro-5-[(2*R*)-2-hydroxypropyl]furan-2-yl}propanoate*; **2**): Colorless oil. $[\alpha]_D^{20} = +9.2$ ($c = 0.55$, MeOH). IR (KBr): 3449, 2972, 2935, 1735, 1461, 1376, 1191, 1092, 1060. ^{1}H- and ^{13}C-NMR: see *Table 2*. ESI-MS: 231 ($[M+H]^+$), 253 ($[M+Na]^+$), 483 ($[2M+Na]^+$), 269 ($[M+K]^+$). HR-ESI-MS: 253.1418 ($[M+Na]^+$, $C_{12}H_{22}NaO_4^+$; calc. 253.1410).

Homononactyl Nonactate (= rac-*(2*R*)-2-{(2*R*,5*S*)-Tetrahydro-5-[(2*S*)-2-{[(2*S*)-2-{(2*S*,5*R*)-tetrahydro-5-[(2*R*)-2-hydroxypropyl]furan-2-yl}propanoyl]oxy}butyl]furan-2-yl}propanoic Acid*; **5**): Colorless oil. $[\alpha]_D^{20} = -7.63$ ($c = 3.10$, MeOH). ^{1}H- and ^{13}C-NMR: see *Tables 3* and *4*. ESI-MS: 401 ($[M+H]^+$), 418 ($[M+NH_4]^+$), 423 ($[M+Na]^+$), 818 ($[2M+NH_4]^+$), 823 ($[M+Na]^+$), 399 ($[M-H]^-$), 799 ($[2M-H]^-$).

Homononactyl Homononactate (= rac-*(2*R*)-2-{(2*R*,5*S*)-Tetrahydro-5-[(2*S*)-2-{[(2*S*)-2-{(2*S*,5*R*)-5-[(2*R*)-tetrahydro-2-hydroxybutyl]furan-2-yl}propanoyl]oxy}butyl]furan-2-yl}propanoic Acid*; **6**): Colorless oil. $[\alpha]_D^{20} = 0$ ($c = 0.80$, MeOH). IR (KBr): 3422, 2968, 2936, 1729, 1462, 1383, 1197, 1064. ^{1}H- and ^{13}C-NMR: see *Tables 3* and *4*. ESI-MS: 415 ($[M+H]^+$), 437 ($[M+Na]^+$), 413 ($[M-H]^-$). HR-ESI-MS 413.2537 ($[M-H]^-$, $C_{22}H_{37}O_7^-$; calc. 413.2539).

Ethyl Homononactyl Nonactate (= *(2*S*)-1-{(2*S*,5*R*)-5-[(2*R*)-1-Ethoxy-1-oxopropan-2-yl]tetrahydrofuran-2-yl}butan-2-yl (2*S*)-2-{(2*S*,5*R*)-Tetrahydro-5-[(2*R*)-2-hydroxypropyl]furan-2-yl}propanoate*; **7**): Colorless oil. $[\alpha]_D^{20} = -12.53$ ($c = 0.76$, MeOH). IR (KBr): 3461, 2971, 2937, 1733, 1460, 1377, 1191, 1091, 1062. ^{1}H- and ^{13}C-NMR: see *Tables 3* and *4*. ESI-MS: 429 ($[M+H]^+$), 446 ($[M+NH_4]^+$), 451 ($[M+Na]^+$), 874 ($[2M+NH_4]^+$). HR-ESI-MS: 451.2680 ($[M+Na]^+$, $C_{23}H_{40}NaO_7^+$; calc. 451.2672).

Ethyl Homononactyl Homononactate (= *(2*S*)-1-{(2*S*,5*R*)-5-[(2*R*)-1-Ethoxy-1-oxopropan-2-yl]tetrahydrofuran-2-yl}butan-2-yl (2*S*)-2-{(2*S*,5*R*)-Tetrahydro-5-[(2*R*)-2-hydroxybutyl]furan-2-yl}propanoate*; **8**): Colorless oil. $[\alpha]_D^{20} = 0$ ($c = 0.25$, MeOH). IR (KBr): 3449, 2969, 2932, 1732, 1459, 1388, 1193, 1066. ^{1}H- and ^{13}C-NMR: see *Tables 3* and *4*. ESI-MS: 443 ($[M+H]^+$), 460 ($[M+NH_4]^+$), 465 ($[M+Na]^+$). HR-ESI-MS: 465.2831 ($[M+Na]^+$, $C_{24}H_{42}NaO_7^+$; calc. 465.2823).

Ethyl Nonactyl Nonactate (= *(2*S*)-1-{(2*S*,5*R*)-5-[(2*R*)-1-Ethoxy-1-oxopropan-2-yl]tetrahydrofuran-2-yl}propan-2-yl (2*S*)-2-{(2*S*,5*R*)-Tetrahydro-5-[(2*R*)-2-hydroxypropyl]furan-2-yl}propanoate*; **9**): Colorless oil. $[\alpha]_D^{20} = 0$ ($c = 0.15$, MeOH). IR (KBr): 3448, 2973, 1734, 1459, 1377, 1192, 1090, 1059. ^{1}H- and ^{13}C-NMR: see *Tables 3* and *4*. ESI-MS: 415 ($[M+H]^+$), 437 ($[M+Na]^+$). HR-ESI-MS: 415.2702 ($[M+H]^+$, $C_{22}H_{39}O_7^+$; calc. 415.2696).

Bishomononactic Acid (= rac-*(2*S*)-2-{(2*S*,5*R*)-Tetrahydro-5-[(2*S*)-2-hydroxy-3-methylbutyl]furan-2-yl}propanoic Acid*; **10**): Colorless oil. ^{1}H-NMR (300 MHz, CD_3OD): 3.96–4.00 (*m*, 1 H); 3.86–3.90 (*m*, 1 H); 3.39–3.45 (*m*, 1 H); 2.32–2.37 (*m*, 1 H); 1.87–1.96 (*m*, 2 H); 1.36–1.65 (*m*, 5 H); 1.01 (*d*, $J = 6.9$, 3 H); 0.80 (*d*, $J = 7.2$, 6 H). ^{13}C-NMR (75 MHz, CD_3OD): 181.6; 82.0; 78.0; 74.6; 47.1; 42.3; 35.3; 32.5; 29.6; 19.2; 17.9; 14.0. ESI-MS: 253 ($[M+Na]^+$), 229 ($[M-H]^-$, 459 ($[2M-H]^-$). The spectral properties were identical to those reported in [6].

Synthesis of **3a** *and* **3b**. *Homononactic acid* (**3**; 18 mg) and L-phenylalanine methyl ester (25 mg) were dissolved in 5 ml of CH_2Cl_2, then 25 mg of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 16 mg of 4-(dimethylamino)pyridine (DMAP) were added, and the mixture was stirred at r.t. for 48 h for the preparation of homonoactic amide derivatives. Completion of the reaction was monitored by TLC. HCl (1N, 10 ml) was added with vigorous stirring to quench the reaction. The org. layer was separated, and the aq. layer was extracted with CH_2Cl_2 (2×10 ml). The combined org. extracts were washed with brine (30 ml) and dried (Na_2SO_4). After filtration and evaporation *in vacuo*, the residue was purified by prep.

HPLC (*YMC Pack ODS-A* (30 × 250 mm, S-15 μm, 12 nm); 70% aq. MeOH soln.; flow rate, 10 ml/min). Collected fractions were evaporated under reduced pressure to afford **3a** (8.5 mg) and **3b** (15 mg).

Data of **3a**. White powder. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.20–7.31 (*m*, 3 H); 7.08–7.14 (*m*, 2 H); 4.90 (*q*-like, *J* = 5.7, 1 H); 4.05–4.08 (*m*, 1 H); 3.74–3.82 (*m*, 1 H); 3.70 (*s*, 3 H); 3.63–3.70 (*m*, 1 H); 3.04–3.18 (*m*, 2 H); 2.33 (*qunint.*-like, *J* = 7.3, 1 H); 1.92–2.02 (*m*, 2 H); 1.64 (*t*, *J* = 5.9, 2 H); 1.56–1.60 (*m*, 2 H); 1.45 (*quint.*-like, *J* = 6.7, 2 H); 1.13 (*d*, *J* = 7.0, 3 H); 0.92 (*t*, *J* = 7.3, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 173.8; 172.2; 136.2; 129.3 (2 C); 128.4 (2 C); 126.9; 80.8; 76.9; 70.3; 53.0; 52.1; 45.8; 41.6; 38.1; 30.6; 30.3; 29.7; 14.3; 9.9. ESI-MS: 378 ($[M+H]^+$), 400 ($[M+Na]^+$).

Data of **3b**. White powder. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.15–7.23 (*m*, 3 H); 7.03 (*d*, *J* = 7.3, 2 H); 4.82 (*q*-like, *J* = 6.2, 1 H); 4.02–4.06 (*m*, 1 H); 3.68–3.76 (*m*, 1 H); 3.65 (*s*, 3 H); 3.48–3.52 (*m*, 1 H); 3.11 (*dd*, *J* = 13.5, 5.9, 1 H); 3.01 (*dd*, *J* = 13.5, 5.9, 1 H); 2.23 (*quint.*-like, *J* = 7.3, 1 H); 1.84–1.93 (*m*, 2 H); 1.31–1.65 (*m*, 6 H); 1.04 (*d*, *J* = 7.0, 3 H); 0.82 (*t*, *J* = 7.3, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 173.8; 172.3; 136.2; 129.2 (2 C); 128.4 (2 C); 126.9; 80.8; 76.8; 69.9; 53.2; 52.2; 45.8; 41.6; 37.9; 30.4; 30.2; 29.8; 13.6; 9.9. ESI-MS: 378 ($[M+H]^+$), 400 ($[M+Na]^+$).

Preparation of L*-Phenylalanine Amide Derivatives of* **3**–**6** *for HPLC Analysis.* In an entirely analogous way as that for the preparation of **3a** and **3b**, with the exception of purification by prep. HPLC, amide derivatives of **3** (2.0 mg), **4** (1.9 mg), **5** (2.5 mg), and **6** (2.5 mg) were obtained for HPLC/MS analysis. Derivatives of **3**–**6** were dissolved in 5 ml of MeOH, resp., and further diluted 1,000 times with MeOH/H_2O 1:1 for HPLC analysis.

Preparation of Amide Derivatives of **1**, **2**, *and* **5** *for HPLC Analysis.* To a soln. of **1** (2 mg) in 95% aq. EtOH (2 ml), 0.1N KOH (0.5 ml) was added. After stirring at r.t. for 24 h, the mixture was adjusted to pH 7–8 with 0.1N HCl soln. and concentrated *in vacuo*. The residue was worked up by dilution with H_2O and extraction with CH_2Cl_2. The org. phase was washed with brine, dried (Na_2SO_4), and evaporated. The residue was taken up in CH_2Cl_2 (2 ml) and treated with an excess of L-phenylalanine methyl ester (5 mg), DCC (3 mg), and DMAP (1.5 mg). After stirring for 48 h at r.t., the mixture was diluted with H_2O and extracted with CH_2Cl_2. The org. phase was washed sequentially with 1N HCl and brine, dried (Na_2SO_4), and evaporated to afford the amide of **1**.

In an entirely analogous way, the amides of **2** and **5** (2 and 2.6 mg, resp.) were prepared. Amides of **1**, **2**, and **5** were dissolved in 5 ml of MeOH, and further diluted 1,000 times with MeOH/H_2O 1:1 for HPLC analysis, resp.

HPLC/MS Analysis of **3a/3b**, **4a/4b**, **5a/5b**, *and* **6a/6b**. *Agilent 1290-6420 Triple Quadrupole* LC/MS system with a *Agilent SB-C18* column (2.1 × 50 mm, 1.8 μm) was used to analyze the L-phenylalanine methyl ester derivatives. A positive SIM (selected ion monitoring) mode was used to analyze **3a**/**3b** (*m*/*z* 400 ($[M+Na]^+$)), **4a**/**4b** (*m*/*z* 386 ($[M+Na]^+$)), **5a**/**5b** (*m*/*z* 584 ($[M+Na]^+$)), and **6a**/**6b** (*m*/*z* 598 ($[M+Na]^+$)). The instrument was operated in positive-ion electrospray ionization mode. The TQ (triple quadrupole)-MS conditions utilized were a gas temp. of 330°, gas flow of 10 l/min, nebulizer pressure of 35 psi, cap. voltage of 4000 V, and cell accelerator voltage of 1 V. The mobile-phase solvent composition was 50% *A* (0.1% HCOOH in H_2O) and 50% *B* (MeOH) for **3a**/**3b**, 55% *A* and 45% *B* for **4a**/**4b**, and 32% *A* with 68% *B* for **5a**/**5b** and **6a**/**6b**. The flow rate was 0.4 ml/min, and the column was heated to 30°.

Cytotoxicity Assays. An MTT assay procedure was used to determine the cytotoxic activities of **1**–**10** against human gastric carcinoma (BGC-823), human hepatocellular liver carcinoma (HepG2), human large-cell lung carcinoma (H460), human colon carcinoma (HCT116), and human cervix carcinoma cell lines (HeLa) [17]. The IC_{50} value was defined as the 50% reduction of absorbance in the control assay. Adriamycin was used as a positive control.

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