Millifolides A–C. New 1,10-Seco-guaianolides from the Flowers of *Achillea millefolium*

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Ten 1,10-secoguaianolides were isolated from the flowers of *Achillea millefolium*. Their structures were determined on the basis of spectroscopic methods. Three of them (millifolides A–C) including two dimeric sesquiterpenoids exhibit new skeletons. Seco-tanapartholide A exhibited moderate cell growth inhibitory activity against the human cancer cell line MCF7WT ($IC_{50} = 5.51\,\mu$m) *in vitro*.

Key words: *Achillea millefolium*, 1,10-Seco-guaianolides, Millifolides A to C, Structure Elucidation, Cell Growth Inhibition

Introduction

The genus *Achillea* comprises more than 100 species distributed in the northern hemisphere, usually represented by small herbs [1]. Aerial parts of different species of this genus are widely used in folk medicine for the preparation of herbal teas with antiphlogistic and spasmolytic activity. A number of sesquiterpene lactones have been reported from this genus, mostly eudesmanolides and guaianolides [2]. *Achillea millefolium* Linnaeas (English name: yarrow), one of the most abundantly occurring species, has played an important role for a long time as a drug in traditional and modern medical practice [3], and its anti-inflammatory properties have been investigated [4]. In a previous phytochemical investigation, as part of our efforts of screening bioactive agents with potential antitumor activities from higher plants, we found that the methanolic extract of *A. millefolium* exhibited a significant cytotoxicity against cultured human tumor cell lines MCF7WT *in vitro*. This paper reports the isolation and structure elucidation of three new and seven known compounds from these extracts.

Results and Discussion

*Isolation and structure elucidation of millifolide A (**1**)*

The methanolic extract of the flower of *Achillea millefolium* was partitioned between hexane and water, and then the aqueous layer was extracted with ethyl

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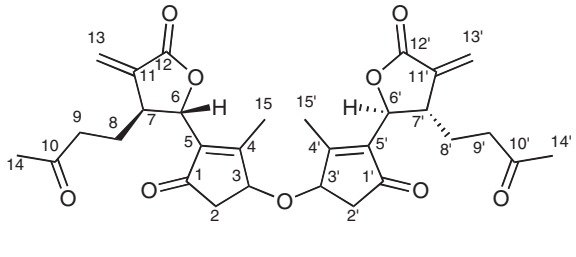
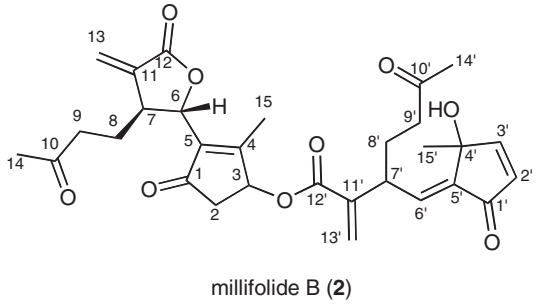
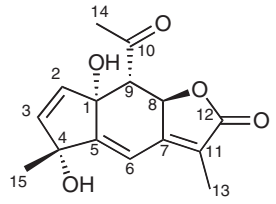


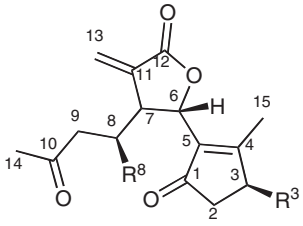
millifolide A (**1**)



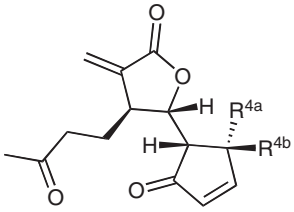
millifolide B (**2**)



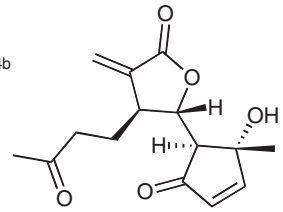
millifolide C (**3**)



	R^3	R^8
4	OH	H
5	H	OH
6	OAc	H
7	OCH_3	H



	R^{4a}	R^{4b}
8	OH	CH_3
9	CH_3	OH



10

Fig. 1. 1,10-Secoguaianolides isolated from *Achillea millefolium*.

acetate. The ethyl acetate-soluble portion was subjected to silica gel column chromatography followed by reversed-phase preparative HPLC to afford compounds **1** to **10** (Fig. 1).

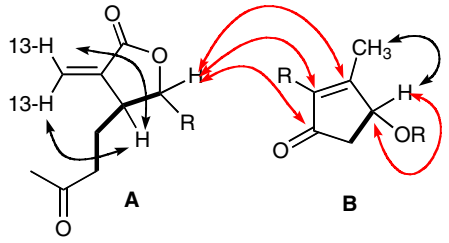


Fig. 2 (color online). Partial structures **A** and **B**. Bold bonds indicate vicinal coupling, black arrows donate long-range ^{1}H-^{1}H COSY, and red arrows show HMBC correlations.

Compound **1** was isolated as an optically active gummy substance. The ^{13}C NMR spectrum of **1** disclosed 15 carbon signals (Table 1). The presence of an α-methylene-γ-lactone moiety was indicated by the characteristic signals at $\delta = 169.6$ (C-12), 137.8 (C-11), 123.0 (C-13), 75.7 (C-6), and 42.7 (C-7). The signals at $\delta = 201.6$, 170.0, 139.0, and 207.4 implied the presence of an α,β-unsaturated group and an unconjugated keto carbonyl unit. Additionally, the ^{13}C NMR spectrum indicated the presence of an oxygenated carbon at $\delta = 77.5$ (C-3), three methylene and two methyl groups and one methine carbon. The ^{1}H NMR spectrum displayed the characteristic signals of exocyclic methylene protons at $\delta = 6.36$ (1H, d, $J = 2.6$ Hz, H-13a) and 5.67 (1H, d, $J = 2.6$ Hz, H-13b), a proton vicinal to the oxygen of the lactone ring at $\delta = 4.96$ (1H, d, $J = 5.0$ Hz, H-6), one allylic methyl group at $\delta = 2.10$ (3H, s, Me-15) placed in the β-position of an α,β-unsaturated keto carbonyl group and a methyl group connected to a keto carbonyl group at $\delta = 2.15$ (3H, s, Me-14). The ^{1}H-^{1}H COSY experiments revealed two sequences of coupled signals from H-6 to H-9 and from H-2 to H-3. Long-range coupling between Me-15 and H-3 as well as between H-7 and H_2-13 were also observed in the ^{1}H-^{1}H COSY spectrum. These findings pointed at the following two partial structures **A** and **B** (Fig. 2).

The HMBC experiments verified the above fragments. The assembly of the two partial structures **A** and **B** is supported by HMBC correlations from H-6 to C-1, C-4 and C-5 and allowed no alternative structure. One interesting feature in the HMBC experiment was that H-3 showed a correlation with C-3. This observation suggested that this compound was a symmetric dimer. This hypothesis was corroborated by the FAB mass spectrum. The molecular formula of **1**, $C_{30}H_{34}O_9$, was calculated by high-resolution molec-

Table 1. The ^{1}H and ^{13}C NMR data for **1** and **2** in $CDCl_3$ (500 MHz for ^{1}H, 125 MHz for ^{13}C).

Position	**1**			**2**		
	δ ^{1}H, mult.[a]	J (Hz)	δ ^{13}C[b]	δ ^{1}H, mult.[a]	J (Hz)	δ ^{13}C[b]
1			201.6			201.9
2a	2.80, dd	18.1, 5.9	41.4	2.93, dd	18.0, 6.3	41.5
2b	2.36, dd	18.1, 2.1		2.34, dd	18.0, 2.3	
3	4.49, br.d	5.9	77.5	5.75, d	6.3	73.4
4			139.0			140.1
5			170.0			168.3
6	4.96, d	5.0	75.7	4.99, d	4.3	75.8
7	3.11, m		42.7			42.9
8a	1.95, m		27.3	1.95, m		27.6
8b	1.87, m			1.84, m		
9a	2.58, m		39.2	2.60, m		39.4
9b	2.55, m			2.53, m		
10			207.4			207.4
11			137.8			138.0
12			169.6			169.7
13a	6.36, d	2.6	123.0	6.36, d	2.5	123.1
13b	5.67, d	2.6		5.67, d	2.5	
14	2.15, s		30.0		2.15	29.9
15	2.10, br.s		14.5		2.09	14.4
1′			201.6			194.8
2′a	2.80, d	18.1, 5.9	41.4	6.28, d	6.0	133.0
2′b	2.36, d	18.1, 2.1				
3′	4.49, br.d	5.7	77.5	7.36, d	6.0	163.7
4′			139.0			75.8
5′			170.0			141.7
6′	4.96, d	5.7	75.7	6.48, d	5.0	135.0
7′	3.11, m		42.7	4.08, m		38.2
8′a	1.95, m		27.3	1.98, m		29.0
8′b	1.87, m			1.88, m		
9′a	2.58, m		39.2	2.46, t	6.9	40.9
9′b	2.55, m					
10′			207.4			209.6
11′			137.8			141.0
12′			169.6			165.8
13′a	6.36, d	2.7	123.0	6.32, s		127.2
13′b	5.67, d	2.4		5.79, s		
14′	2.15, s		30.0	2.11, s		29.9
15′	2.10, br.s		14.5	1.57, s		26.0

[a] Mult. (multiplicity): s, singlet; d, doublet; t, triplet; dd, doublet of doublet; br, broadened; m, multiplet. The precision of the coupling constants is ±0.5 Hz; [b] the ^{13}C chemical shifts were extracted from the HSQC and HMBC experiments (±0.2 ppm).

ular mass measurement on the basis of the ion at $m/z = 577.1836$ $[M+K]^+$. Therefore, the structure of **1** was established to be a dimeric ether dehydrative of *iso*-seco-tanapartholide (**4**) isolated from the same origin [5]. Since the ^{1}H NMR spectral data of **1** resembled those of **4**, the relative configuration of *trans*-C-6,7 and C-3 is as shown. The *trans*-substitution pattern of the γ-lactone ring was verified by NOE correlations of H2b/H7, H6/H8a, H6/H8b, and H6/H9. Its optically active nature indicated that **1** does not display a *meso*-form. The absolute configuration of **1** is assumed to be the same as that of **4**. Compound **1** represents the first example of a dimeric 1,10-secoguaianolide from a natural source, and is named millifolide A [6].

*Structure elucidation of millifolide B (**2**)*

The molecular formula of **2** was deduced from the high-resolution FAB mass spectrum as $C_{30}H_{34}O_9$, which was exactly the same as that of **1**. The ^{13}C NMR spectrum (Table 1) displayed 30 carbon signals including two α,β-unsaturated keto carbonyl signals

at $\delta = 201.9$ (C-1) and 194.8 (C-1′), two unconjugated keto carbonyl signals at $\delta = 207.4$ (C-10) and 209.6 (C-10′), two ester carbonyl carbon signals at $\delta = 169.7$ (C-12) and 165.8 (C-12′), five pairs of C=C bond signals at $\delta = 138.0$ (C-11), 123.1 (C-13), 168.3 (C-4), 140.1 (C-5), 141.0 (C-11′), 127.2 (C-13′), 133.0 (C-2′), 163.6 (C-3′), 141.7 (C-5′), and 135.0 (C-6′), and three oxygenated carbon signals at $\delta = 75.8$ (C-6), 73.4 (C-3) and 75.8 (C-4′). When comparing the ^{13}C NMR spectra of **1** and **2**, all signals of **1** could be found in **2** except that the signals of C-3, C-4 and C-5 of **2** were shifted by +4.1, −1.1 and +1.7 ppm, respectively. In the ^{1}H NMR spectrum, the most distinguished difference was shown by the carbinol proton H-3 which was shifted from $\delta = 4.47$ in **1** to $\delta = 5.75$ in **2**. This observation suggested that the two partial structures were asymmetrically connected not by an ether but by an ester linkage. Indeed, the resemblance of the ^{1}H and ^{13}C NMR signals of the right hand part indicated that ester **2** was composed of achimillic acid A [7] and *iso*-seco-tanapatholide (**4**). The connection was elucidated by the crucial long-range proton-carbon correlation between H-3 and C-12′ in the HMBC experiment. The relative configurations of C-3, C-6 and C-7 are assumed to be according to the congeners **4**, **8**, **9**, and **10** isolated together with **2**. This was confirmed by the ROE correlations of H2b/H7, H6/H8a, H6/H8b, H6/H9a, and H6/H9b. Those of C1′, C6′ and C7′ were verified by the ROE correlation of H7′/H15′. Thus, the structure of **2**, named millifolide B, has been characterized as shown.

*Structure elucidation of millifolide C (**3**)*

Compound **3** was obtained as a gummy substance. The FABMS of **3** displayed a pseudo-molecular ion peak at $m/z = 299$ $[M+Na]^+$, and a high-resolution peak at $m/z = 299.0880$ established its elemental composition as $C_{15}H_{16}O_5$. The ^{13}C NMR (Table 2) spectrum accounted for the presence of 15 carbon atoms in the molecule, of which those resonating at $\delta = 173.9$ and $\delta = 210.8$ were assignable to an α,β-unsaturated lactone (C-12) and a keto (C-10) carbonyl group, respectively. Three tri-substituted and three fully substituted olefinic carbons were found at low field [$\delta = 133.1$ (C-2), 141.6 (C-3), 112.1 (C-6), 158.8 (C-5), 154.5 (C-7) and 121.5 (C-11)]. Three resonances at $\delta = 60.4$ (C-1), 80.3 (C-4) and 76.9 (C-8) could be attributed to oxygenated carbons. Two of them corresponded to the fully substituted carbons (C-1 and C-4) and one to the methine carbon (C-8). A combined HMBC and HMQC experiment established that the ^{13}C NMR spectrum was composed of one oxymethine, one sp^3 methine, two sp^3 quaternary carbons, and three methyl groups indicating that **3** contains 15 carbon and 14 carbon-bonded hydrogen atoms. Thus, **3** contains two hydroxy groups. Since 5 out of 8 unsaturations were accounted for by three double bonds, one ketone and one lactone, **3** was inferred to contain three rings. Data from ^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY experiments were used to generate the two partial structures **C** and **D** (Fig. 3) for **3**. From the HMBC and ^{1}H-^{1}H COSY spectra the partial structure **C** containing a cyclopen-

Table 2. The ^{1}H and ^{13}C NMR data for **3** in $CDCl_3$ (500 MHz for ^{1}H, 125 MHz for ^{13}C).

Position	δ ^{1}H, mult.	J (Hz)	δ ^{13}C	HMBC[a]
1			80.4	
2	6.02, d	6.0	133.1	1/4, 3, 5
3	6.07, d	6.0	141.6	2/5, 1, 4, 15, (6)
4			80.3	
5			158.8	
6	6.69, s		112.1	1/4, 5, 7, 8, (9, 12, 15)
7			154.5	
8	5.49, dq	11.6, 1.3	76.9	7
9	2.68, d	11.6	59.6	1, 7, 8, 10, 14
10			210.8	
11			121.5	
12			173.9	
13	2.43, s		32.9	7, 11, 12 (5, 6, 8)
14	1.96, d	1.3	87.4	9
15	1.61, br.s		27.8	1, 3, 5
1-OH	4.17, br.s			1, 5, 9

[a] Numbers in parentheses show weak correlations (through 4 or 5 bonds).

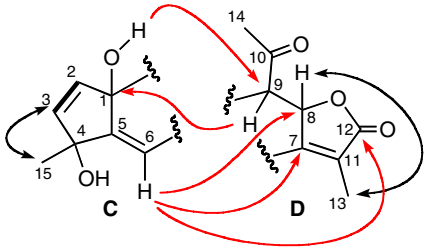


Fig. 3 (color online). Partial structures **C** and **D** of **3**. Bold bonds indicate vicinal coupling, black arrows donate long-range ^{1}H-^{1}H COSY, and red arrows show HMBC correlations.

tene skeleton with two tertiary hydroxy groups was deduced.

The sharp three-proton singlet at $\delta_{\text{H-14}} = 2.43$, together with the carbonyl peak at $\delta_{\text{C}} = 210.8$, indicated the presence of a methyl ketone (-COMe) moiety, which was supported by FAB-MS $[\text{M–COCH}_3]^+$. The signal at $\delta_{\text{H}} = 2.68$, which showed long-range correlation with $\delta_{\text{C-14}} = 32.9$ and $\delta_{\text{C-10}} = 210.8$ in the HMBC experiment, was assigned to H-9 in α-position to the carbonyl group. The ^{1}H-^{1}H COSY spectrum showed a correlation between $\delta_{\text{H-9}} = 2.68$ and $\delta_{\text{H}} = 5.49$. The appearance at lower field combined with its corresponding resonance in the ^{13}C NMR spectrum at $\delta_{\text{C-8}} = 76.9$ indicated that the signal at $\delta_{\text{H}} = 5.49$ was a γ-lactonic methine. A decoupling experiment revealed a homoallylic relationship between H-8 and H_3-14 ($^{5}J = 1.3$ Hz). Thus, the partial structure **D** was deduced from ^{13}C NMR and HMBC spectral information as an α-methyl-α,β-unsaturated-γ-butenolide ring ($\delta_{\text{H-13}} = 1.96$, *d*, $J = 1.3$ Hz, $\delta_{\text{C-13}} = 87.4$, $\delta_{\text{C-11}} = 121.5$, $\delta_{\text{C-7}} = 154.5$, $\delta_{\text{C-12}} = 173.9$, and $\delta_{\text{C-8}} = 76.9$). The vicinal H-H coupling constant between H-8 and H-9 ($J = 11.6$ Hz) showed their *trans*-diaxial relationship. Two- and three-bond ^{1}H-^{13}C correlations were used to connect these two partial structures **C** and **D** (Fig. 4) as well as to confirm the above structural assignment. Critically, H-6 in **C** showed correlations with C-7, C-8 and C-12 of **D**. Furthermore, the tertiary hydroxy proton at C-1 in **C** exhibited a correlation with C-9 in **D**; in turn, H-9 in **D** correlated with C-1 in **C**. Therefore, the partial structures **C** and **D** were connected through C-6/C-7 and C-1/C-9. This combination is reasonable from the viewpoint of biogenetic considerations. Compound **3** is most probably derived from a *pseudo*-guaianolide precursor **E** (Scheme 1). Oxidative cleavage of the five-membered ring in **E**

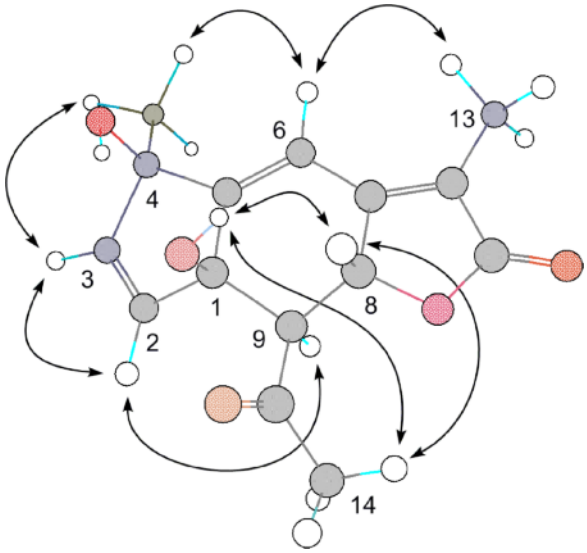
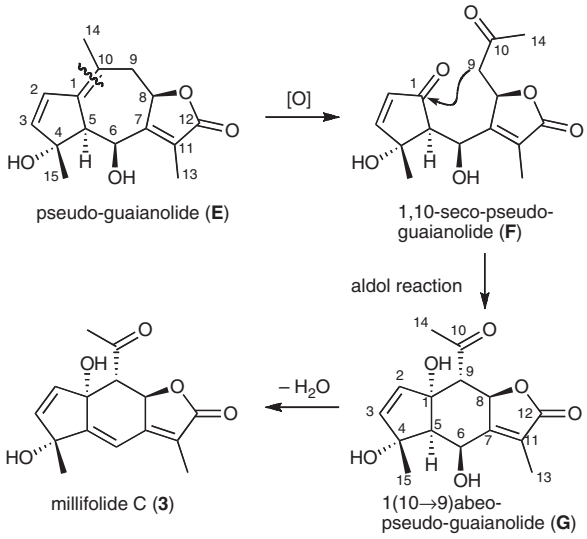


Fig. 4 (color online). Key NOEs of **3** obtained from the NOESY experiment. A conformational search was carried out by minimizing the energy using standard MM2 constants based on the structure elucidated by the NOESY data [CHEM3D (ver. 10), Cambridge-Soft, Cambridge, MA (USA)].



Scheme 1. Proposed biosynthetic route to **3** starting from a pseudo-guaianolide.

followed by intramolecular aldol reaction lead to **G**, which loses one water molecule affording **3** [8].

The relative stereochemical assignments were accomplished by extensive NOESY experiments and

Table 3. The ^{1}H data for **4**–**10** in $CDCl_3$ (500 MHz)[a].

Position	**4**	**5**	**6**	**7**	**8**	**9**	**10**
2a	2.81, dd	2.38, m	2.88, m	2.67, dd	6.09, d	6.12, d	6.07, d
2b	2.32, dd		2.28, m	2.31, dd			
3	4.71, br.d	2.58, m	5.66, m	4.29, br.d	7.49, d	7.43, d	7.47, d
5					2.52, m	2.29, d	2.68, d
6	4.96, d	5.24, br.d	4.98, d	4.97, d	4.55, m	4.55, m	4.46, dd
7	3.08, m	3.26, m	3.12, m	3.05, m	3.64, m	3.49, m	3.45, m
8a	1.96, m	4.27, dt	1.86, m	1.96, m	1.91, m	1.85, m	1.94, m
8b	1.87, m		1.84, m	1.85, m			
9a	2.56, m	2.65, m	2.60, m	2.58, dt	2.53, m	2.53, m	2.64, dt
9b			2.53, m	2.51, ddd			2.57, ddd
13a	6.34, d	6.41, d	6.36, d	6.33, d	6.25, d	6.30, br.s	6.36, d
13b	5.66, d	5.73, d	5.67, d	5.65, d	5.61, d	5.70, br.s	5.77, d
14	2.14, s	2.20, s	2.15, s	2.13, s	2.15, s	2.14, s	2.20, s
15	2.16, s	2.15, br.s	2.09, s	2.13, s	1.51, s	1.56, s	1.58, s
3-OAc			2.12, s				
3-OMe				3.40, s			

[a] Multiplicities: *J* (Hz). **4**: H-2a = 18.0, 6.0; H-2b = 18.0, 2.2; H-3 = 6.5; H-6 = 5.3; H-13a = 2.8; H-13b = 2.4. **5**: H-6 = 3.7; H-8 = 7.0, 5.3; H-13a = 2.4; H-13b = 2.2. **6**: H-6 = 4.8; H-13a = 2.8; H-13b = 2.4. **7**: H-2a = 18.2, 6.0; H-2b = 18.2, 2.0; H-3 = 5.8; H-6 = 5.0; H-9a = 18.1, 7.5; H-9b = 18.1, 8.1, 5.8; H-13a = 2.6; H-13b = 2.3. **8**: H-2 = 5.8; H-3 = 5.8; H-13a = 2.7; H-13b = 2.4. **9**: H-2 = 5.6; H-3 = 5.6; H-5 = 8.6. **10**: H-2 = 5.8; H-3 = 5.8; H-5 = 10.6; H-6 = 10.6, 2.1; H-9a = 17.6, 7.5; H-9b = 17.6, 7.8, 5.9; H-13a = 1.9; H-13b = 1.7.

molecular modeling studies (Fig. 4). The observation of a strong NOE between H-14 and H-8 allowed the determination of the relative stereochemistry at the C-9 acetyl group in the α-equatorial and H-8 in the α-pseudo-equatorial positions. This assignment is in good agreement with the *trans*-diaxial relationship between H-8 and H-9 (J = 11.6 Hz). The data cited above enable to establish the structure of **3** as (9βH)-1α,4α-dihydroxy-10-oxo-1,10-seco-1(10 → 9)*abeo*guaia-2,5,7(11)-trien-12,8β-olide, being named millifolide C. Compound **3** represents a new rearranged guaiane skeleton.

Isolation of other derivatives

Other compounds were characterized as *iso*-secotanapartholide (**4**) [5], arteludooicinolide A (**5**) [9], 3-acetyl-*iso*-seco-tanapartholide (**6**) [9c], 3-methoxy-tanapartholide (**7**) [9c, 10], seco-tanapartholide A (**8**), seco-tanapartholide B (**9**) [11], and 5-*epi*-seco-tanapartholide A (**10**) [12] on the basis of 1D and 2D NMR spectroscopic analysis and by comparison with data reported in the literature. Since the NMR spectroscopic assignments of these secoguaianolides has been discussed controversially in the literature, we summarized their ^{1}H and ^{13}C NMR data assigned according to 2D NMR analyses in Tables 3 and 4. Compound **8** exhibited moderate cell growth inhibitory activity against the human cancer cell line MCF7WT (IC_{50} = 5.51 μm) and almost no activity against PC3 (IC_{50} > 100 μm) *in vitro*.

Table 4. The ^{13}C data for **4**–**10** in $CDCl_3$ (125 MHz).

Position	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	202.9	207.5	201.8	202.6	204.9	204.7	202.3
2	44.2	34.3	41.6	40.6	133.3	132.9	141.1
3	71.5	32.4	72.7	79.6	167.2	165.8	166.0
4	172.4	175.0	168.5	138.6	77.2	78.0	79.2
5	137.9	137.5	139.8	171.2	56.7	58.1	62.8
6	75.8	72.3	75.8	75.8	79.7	80.6	80.1
7	43.1	47.9	42.8	43.1	39.8	41.0	41.8
8	27.2	68.9	27.5	27.4	26.2	28.3	28.4
9	39.4	44.7	39.2	39.4	39.4	39.5	39.6
10	207.6	209.5	207.3	207.4	207.3	207.7	207.6
11	138.7	135.3	137.9	138.1	137.9	137.9	138.0
12	169.9	170.3	169.9	169.9	169.3	169.5	169.3
13	122.7	124.0	122.8	122.9	122.6	124.1	124.7
14	29.9	30.8	29.8	29.8	30.0	29.9	29.8
15	14.1	17.4	14.2	14.3	27.2	28.7	25.5
3-OAc			20.7				
			170.5				
3-OMe				57.6			

Conclusion

In conclusion, ten 1,10-secoguaianolides including three new compounds (**1**, **2** and **3**) were isolated from the flowers of *Achillea millefolium*. Compounds **1**

and **2** represent the first examples of dimeric 1,10-secoguaianolides from natural sources, and **3** is a guaianolide with a rare rearranged carbon skeleton. Compound **8** exhibited moderate cell growth inhibitory activity against MCF7 human cancer cells. The *A. millefolium* group is actually divided into more than 10 well-defined species, which are characterized by morphological and anatomical features. However, high biodiversity and naturally occurring hybrids obviously complicate the clear classification of plant individuals. According to the generally accepted biogenetic pathway of sesquiterpenoids, the biogenesis of sesquiterpenoids might be controlled by distinct gene loci. Sesquiterpene patterns serving as excellent chemotaxonomic tools have been reported. Secoguaianolides are mainly occurring in the *Achillea* and *Artemisia* genus of the Asteraceae (Compositae) family. Isolation of ten 1,10-secoguaianolides from the aerial parts of *A. millefolium* will contribute to the differentiation of these species [13].

Experimental Section

General

Optical rotations were measured with a JASCO DIP-370 digital polarimeter. NMR spectra were taken on a Bruker Avance-500 spectrometer (500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C) in $CDCl_3$; the solvent residual peak ($CHCl_3$) was used as a reference (7.25 ppm for ^{1}H and 77.0 ppm for ^{13}C). For phase-sensitive 2D experiments (ROESY and HSQC), the data were acquired using the TPPI phase mode. Two mixing times were used in the ROESY experiment: 0.3 and 0.5 s. FABMS were obtained with a Vacuum Generators ZAB-HS double-focusing instrument using a xenon beam of 8 kV at 1 mA equivalent neutral current. The HR-FABMS was obtained in glycerol-DMSO at a resolving power of 12 000 or with a Micromass MALDIQ-TOF intrument in an α-cyano-4-hydroxycinnamic acid matrix in W-mode with a resolving power of 15 000. Column chromatography was performed on silica gel 60 (230 – 400 mesh EM Science). Thin layer chromatography was conducted on silica gel 60 F254 pre-coated TLC plates (0.25 mm or 0.5 mm, EM Science). The compounds were visualized on TLC plates with 10 % sulfuric acid in ethanol and heating on a hot plate. Na_2SO_4 was the drying agent used in all work-up procedures. Preparative HPLC were carried out on a Waters Delta Prep 3000 instrument coupled to a UV 486 tunable absorbance detector (220 nm, Waters, Montreal, Quebec, Canada) with one partisil 10 ODS-2 MAG-20 column (22 × 500 mm), eluted with a linear gradient of CH_3CN in water from 25 % to 100 % in 50 min (18 mL min^{-1}).

Plant material

The flowers of *Achillea millefolium* were purchased in September, 2003 in Montreal (Quebec, Canada). The plant material was identified by Dr. J. H. Wang of the laboratory of pharmacognosy at Hebei Medical University. Several specimens have been deposited in our laboratory at Hebei Medical University (voucher number Qw-1999-01).

Extraction and isolation

Powdered air-dried flowers of *Achillea millefolium* (4.2 kg) were extracted with 16 L of MeOH by shaking for 1 d at room temperature [14]. The ground plants were filtered and extracted again with fresh solvent (6 L of MeOH) for another 3 d. The combined organic extracts were evaporated under reduced pressure. Water (4 L) was added to the crude extract, and lipids were removed by stirring the mixture with hexane (4 × 3.2 L). The aqueous phase was then salted with NaCl and extracted with EtOAc (4 × 3.2 L). The combined EtOAc extract was dried over anhydrous sodium sulfate, filtered and evaporated to yield 149 g of a dark-brown extract. This EtOAc extract was subjected to dry column chromatography (CC) on silica gel (1.5 kg, 8 × 68 cm bed size), eluting with EtOAc-MeOH (95 : 5, 2.5 L). The silica gel was cut into 15 equal bands after elution, and each band was individually eluted with EtOAc-MeOH (1 : 1). The eluates of the column from bands 3 to 6 were combined after TLC analysis and evaporated to yield 43 g of residue A, which was then fractionated by CC on silica gel (1.2 kg, 9.5 × 30 cm bed size) with hexane-EtOAc (3 : 1 – 1 : 4) as the eluent, affording ten fractions ($A_1 - A_{10}$). Fraction A_3 was further purified by CC (hexane-acetone = 5 : 4) to give fractions $A_{3\text{-}1} - A_{3\text{-}5}$. A part of fraction $A_{3\text{-}2}$ was separated with preparative TLC (CH_2Cl_2/CH_3CN) and further purified with (RP) preparative HPLC. The material eluted at R_t = 24.0 min was collected and concentrated to afford **6** (3 mg). A part of fraction A_4 was subjected to CC (hexane-EtOAc = 1 : 1, 2 : 3 and 1 : 2). One of the sub-fractions, $A_{4\text{-}2}$, was separated with preparative HPLC and further purified with preparative TLC (hexane-acetone = 3 : 2), and finally afforded 2.4 mg of **7** (R_t = 27.2 min, R_f = 0.34). Fraction $A_{4\text{-}3}$ was purified by preparative HPLC and yielded **8** (2.2 mg, R_t = 14.4 min) and **9** (1.8 mg, R_t = 14.7 min). Fraction A_5 was applied to CC (hexane-EtOAc = 1 : 1 to 1 : 2) to afford fractions $A_{5\text{-}1}$, $A_{5\text{-}2}$ and $A_{5\text{-}3}$. A part of fraction $A_{5\text{-}3}$ was separated by RP preparative HPLC followed by preparative TLC (hexane-acetone = 11 : 10) to yield **5** (4 mg, R_t = 13.7 min, R_f = 0.43 hexane-acetone 1 : 1; R_f = 0.60, CH_2Cl_2-$CNCH_3$ = 7 : 3). Fraction A_6 was further fractionated by CC (hexane-acetone =

2 : 1 to 1 : 2) to afford 6 sub-fractions $A_{6\text{-}1}$ to $A_{6\text{-}6}$. A part of fraction $A_{6\text{-}3}$ was submitted to repeated preparative TLC (hexane-EtOAc = 2 : 5 and hexane-acetone = 5 : 4) yielding **10** (1.5 mg, $R_f = 0.28$) and **3** (2 mg, $R_f = 0.34$). A part of $A_{6\text{-}4}$ was applied to a preparative TLC (hexane-acetone = 1 : 2). The band with a $R_f = 0.6$ was cut off and purified by RP preparative HPLC. The material eluted at $R_t = 13.5$ min was collected and concentrated to afford **4** (4 mg). Fraction $A_{3\text{-}1}$ was fractionated by CC (hexane/acetone) to give three sub-fractions $A_{3\text{-}1\text{-}1}$ to $A_{3\text{-}1\text{-}3}$. $A_{3\text{-}1\text{-}3}$ was further purified with preparative TLC and RP HPLC. The materials eluted at $R_t = 27.4$ and 30.0 min were collected and concentrated to afford **2** (2 mg) and **1** (1.5 mg).

*Millifolide A (**1**)*

Gum; $[\alpha]_D^{22} = +23$ ($c = 0.10$, $CHCl_3$). – HMBC: H/C = 2*a*/1, 2*a*/4, 2*a*/5, 2*b*/1, 2*b*/3, 3/3′, 3/4, 3/5, 6/1, 6/4, 6/5, 6/7, 6/8, 8*a*/6, 8*a*/7, 8*a*/9, 8*a*/10, 8*a*/11, 9/7, 9/8, 9/10, 13*a*/11, 13*a*/12, 13*b*/7, 13*b*/11, 13*b*/12, 14/9, 14/10, 15/1, 15/3, 15/4, 15/5; NOESY H/H = 2*a*/3, 2*b*/3, 2*b*/7, 2*b*/15, 3/15, 6/7, 6/8*a*, 6/8*b*, 6/9*ab*, 6/15, 7/8*a*, 7/8*b*, 7/9*ab*, 7/13*b*, 7/15, 8*a*/13*b*, 8*b*/9*ab*, 9*ab*/13*b*, 9*ab*/14, 13*a*/13*b*. – HRMS ((+)-FAB): m/z = 577.1835 (calcd. 577.1834 for $C_{30}H_{34}O_9K^+$, $[M+K]^+$).

*Millifolide B (**2**)*

Gum; $[\alpha]_D^{22} = +15$ ($c = 0.10$, $CHCl_3$). – HMBC: H/C = 2*a*/1, 2*a*/4, 2*b*/1, 2*b*/3, 3/4, 3/5, 3/12′, 6/4, 6/5, 6/7, 6/8, 6/10, 6/12, 9/7, 9/8, 9/10, 13*a*/7, 13*a*/8, 13*a*/11, 13*a*/12, 13*b*/7, 13*b*/12, 14/9, 14/10, 15/1, 15/3, 15/4, 15/5, 2′/1′, 2′/3′, 2′/5′, 3′/1′, 3′/2′, 3′/4′, 3′/5′, 3′/15′, 6′/1′/6′/2′, 6′/4′, 6′/5′, 6′/7′, 6′/8′, 7′/5′, 7′/6′, 7′/8′, 7′/9′, 7′/11′, 7′/12′, 7′/13′, 13′*a*/7′, 13′*a*/11′, 13′*a*/12′, 13′*b*/7′, 13′*b*/12′, 14′/9′, 14′/10′, 15′/3′, 15′/4′, 15′/5′. – ROESY: H/H = 2*a*/3, 6/7, 6/8*a*, 6/8*b*, 6/9*a*, 6/9*b*, 7/13*b*, 8*a*/13*b*, 13*a*/13*b*, 2′/3′, 3′/15′, 6′/7′, 6′/8′*b*, 6′/13′*b*, 7′/8′*a*, 7′/8′*b*, 7′/9′, 7′/13′*b*, 7′/15′, 8′*a*/13′*b*, 13′*a*/13′*b*. – HRMS ((+)-FAB): m/z = 577.1835 (calcd. 577.1834 for $C_{30}H_{34}O_9K^+$, $[M+K]^+$).

*Millifolide C (**3**)*

Gum; $[\alpha]_D^{22} = +13$ ($c = 0.10$, $CHCl_3$). – HRMS ((+)-FAB): m/z = 299.0880 (calcd. 299.0890 for $C_{15}H_{16}O_5Na^+$, $[M + Na]^+$).

Biological evaluation

Human tumor cell lines used were MCF7WT (human breast cancer cell line) and PC3 (human prostatic cancer cell line). The cells were cultured in Eagle's minimal essential medium (EMEM) (GIBCO/BRL, Grand Island, NY, USA), containing 10 % (v/v) calf serum (Intergen, Purchase, NY, USA) and antibiotics (100 μg/mL of streptomycin and 100 units/mL of penicillin G) (Meiji Seika, Tokyo, Japan), at 37 °C in a humidified atmosphere containing 5 % CO_2. Cell survival was estimated by the MTT assay as described [15]. Briefly, logarithmically proliferating cells were plated into 96-well plates (1×10^4 cells per well) with the medium containing the test compounds with the indicated doses, followed by culturing for 2 d. Then, the activity of mitochondrial succinic dehydrogenase was measured by further incubation of the cells with 0.5 mg/mL MTT (Sigma) for 4 h, followed by estimation of the absorbance at 570 nm with a reference wavelength of 655 nm. Cell viability was calculated from the absorbance as percentage of the survived cells.

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