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Diterpenoids from *Isodon pharicus*

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ABSTRACT

A phytochemical investigation of *Isodon pharicus* led to the isolation of a novel asymmetric *ent*-kauranoid dimer, bispseurata F (**1**), and three new diterpenoids, pharicinins **A**–**C** (**2**–**4**). Their structures were elucidated by extensive spectroscopic analysis. Compound **1** features a unique linkage pattern of C-17 with C-11′ to connect the two monomers. A possible biogenetic pathway of **1** was also proposed. Compounds **3** and **4** exhibited moderate inhibitory activity against NB4 and SH-SY5Y cell lines.

Isodon species are well known to contain structurally diverse and bioactive diterpenoids.[1] Over the past 30 years, more than 50 *Isodon* species have been phytochemically investigated, and a large number of new *ent*-kauranoids, including dimeric diterpenoids with six kinds of linkage pattern, have been found by our group.[2–7] However, the dimeric diterpenoid, formed by the Michael addition reaction and connected by the C-17–C-11′, has never been reported.

Isodon pharicus(Prain) Hara, mainly distributed in the northwest of Sichuan Province and the southern district of Tibet Autonomous region, People's Republic of China, has been used for killing roundworm and treatment of inflammation of the eyes.[8a] Previous studies on this plant resulted in the isolation of six *ent*-kuaranoids, including a dimeric diterpenoid.[8b–d] In our search for biologically active secondary metabolites from *Isodon* genus, a novel dimeric *ent*-kauranoid, bispseurata F (**1**), and three new diterpenoids, pharicinins **A**–**C** (**2**–**4**), were isolated from the aerial parts of *I. pharicus*, collected in Lhasa Prefecture, Tibet Autonomous region. Compound **1** was characterized with a unique C-17–C-11′ connection pattern to link the two monomers together, which was proposed to be formed by the Michael addition reaction. In this Letter, we present the isolation and structure elucidation of these new diterpenoids and their cytotoxicity evaluation, as well as the hypothetically biogenetic pathway of **1**.

The aerial parts of *I. pharicus* (7.0 kg) were extracted with 70% aqueous acetone (3 × 40 L) at room temperature overnight. The extract was partitioned between EtOAc and H_2O, and an EtOAc extract (273 g) was chromatographed over a silica gel, eluted in a step gradient manner with $CHCl_3$–CH_3COCH_3 (1:0 to 0:1) to afford fractions A–F. Fraction C (12 g) was submitted to chromatography over a silica gel (petroleum ether–acetone, from 9:1 to 1:1), to give fractions C1–C4. Fraction C2 (850 mg) was purified by repeated chromatography over a silica gel (petroleum ether–acetone, from 40:1 to 1:1) and RP-18 column (32% MeOH–H_2O), to yield compound **1** (10 mg). Fraction B (42 g) was submitted to chromatography over a silica gel (petroleum ether–acetone, from 99:1 to 1:1), to give fractions B1–B5. Compounds **2** (23 mg) and **4** (5 mg) were acquired from fraction B2 (1.2 g), and compound **3** (7 mg) was obtained from fraction B4 (3.8 g) by RP-18 (40% MeOH–H_2O).

Bispseurata F (**1**), isolated as a white, amorphous powder, exhibited the molecular formula $C_{44}H_{60}O_{14}$ on the basis of the positive HRESIMS (*m/z* 835.3900 $[M+Na]^+$, calcd 835.3880), corresponding to 15 degrees of unsaturation.[9] The IR absorptions at 3430, 1731, and 1643 cm^{-1} indicated the presence of hydroxyl, carbonyl, and α,β-unsaturated ketone groups, respectively. The ^1H, ^{13}C, and DEPT NMR spectra (Table 1) displayed 44 carbon resonances due to eight methyls (of which two belonged to acetyls), seven methylenes (including one olefinic methylene), sixteen methines (of which eight were oxygenated), and thirteen quaternary carbons (including one olefinic carbon and six carbonyls). Above evidences showed that **1** could be a dimeric diterpenoid.

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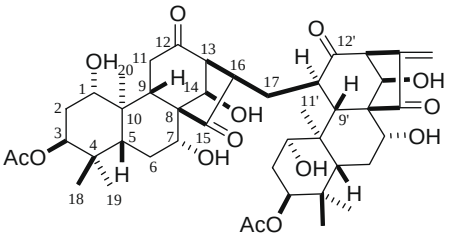

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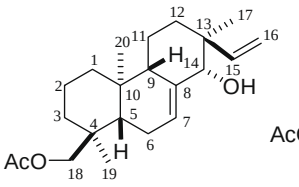

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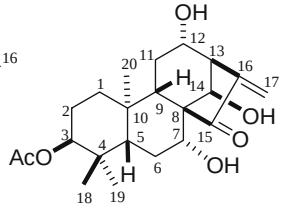

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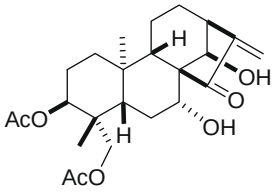

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Comparison of the NMR data of **1** with those of pseurata F[10], the monomer of **1**, also isolated from this plant, revealed that the two diterpenoid units of **1** were **1a** and **1b** (Fig. 1). The ^{1}H and ^{13}C NMR spectra of both **1a** and **1b** resembled those of pseurata F except that the α,β-unsaturated ketone in pseurata F was replaced by an isolated ketone [δ_C 218.7 (s, C-15)], a methine [δ_H 3.20, m (H-16), δ_C 50.3 (d, C-16)], and a methylene [δ_H 1.12, m (H_2-17), δ_C 35.7 (t, C-17)] in **1a**, while a methine [δ_H 4.05, m (H-11′), δ_C 48.9 (d, C-11′)] in **1b** substituted for a methylene [δ_C 38.7 (t, C-11)] in pseurata F. All these key changes of the characteristic signals were suggestive that the subunits **1a** and **1b** are connected by a single carbon-carbon bond (C17–C11′), which was proven by the ^{1}H–^{1}H COSY correlations of H-14/H-13/H-16/H_2-17/H-11′/H-9′, as well as the observed HMBC correlations from H_2-17 to C-13 (δ_C 60.4), C-15 (δ_C 218.7), C-16 (δ_C 50.3), C-9′ (δ_C 53.0), and C-12′ (δ_C 209.0) (Fig. 1). In addition, since the correlations observed in the HMBC spectrum of subunits **1a** and **1b** were consistent with those of pseurata F, this confirmed the deduction above.

The relative configuration of compound **1** was established on the basis of the ROESY correlations of H-11′ with H-13α, H-16, and Me-20′, of H-17a with H-9′β, and of H-13α with H-14α as shown in the computer-generated 3D drawing (Fig. 1). In addition, the other correlations observed in the HMBC and ROESY spectra of **1** were in good agreement with those of pseurata F, this confirmed that the compound **1** was bispseurata F as shown.

Pharicinin A (**2**) was obtained as a white powder, and its molecular formula $C_{22}H_{34}O_3$ was determined by the positive HRESIMS (*m/z* 369.2404 $[M+Na]^+$, calcd 369.2405), with 6 degrees of unsaturation.[11] Apart from the resonances of an acetyl, twenty carbon signals corresponding to three tertiary methyls, eight methylenes (including one oxygenated and one sp^2 methylene), five methines (including one oxygenated and two sp^2 methines), and four quaternary carbons (including one sp^2 carbon) were observed in the NMR spectra, which were characteristic signals of an isopimarane diterpenoid. Considering from the biogenetic pathway and the fact that compound **2** was isolated from *Isodon* genus, compound **2** should be an *ent*-isopimarane diterpenoid.

The HMBC correlations from H-14 (δ_H 4.02) to C-7 (δ_C 119.0), C-12 (δ_C 35.5), C-15 (δ_C 149.6), and C-17 (δ_C 15.9) and from H_2-18 (δ_H 3.80 and 3.92) to C-3 (δ_C 36.5), C-5 (δ_C 44.7), C-19 (δ_C 18.2), and OAc (δ_C 170.9) suggested that a hydroxyl group and an acetoxy group were attached to C-14 and C-18, respectively. The HMBC correlations of H-15 (δ_H 6.30) with C-12 (δ_C 35.5), C-13 (δ_C 43.1), C-14 (δ_C 77.3), and C-17 (δ_C 15.9), of H_2-16 (5.10 and 5.19) with C-13 (δ_C 43.1), C-14 (δ_C 77.3), and C-17 (δ_C 15.9), and of H-7 (δ_H 6.33) with C-6 (δ_C 23.6), C-9 (δ_C 51.9), and C-14 (δ_C 77.3) indicated that the two double bonds were located at C7-C8 and C15-C16, as shown in Figure 2. In the ROESY spectrum of **2**, H-14 correlated to H-9β and H-11β, and Me-17 correlated to H-11α. These observations indicated that H-14 adopt a β-orientation, while Me-17 is α-oriented. Therefore, **2** was elucidated as 14α-hydroxy-18-acetoxy-*ent*-isopimar-7,15-diene.

The molecular formula ($C_{22}H_{32}O_6$) of pharicinin B (**3**)[12] was deduced from its HRESIMS (*m/z* 415.2089 $[M+Na]^+$). The ^{1}H and ^{13}C NMR data revealed that compound **3** resembled pseurata C[13] with the presence of a hydroxyl at C-12 in **3** rather than a carbonyl at the same position in pseurata C, which was supported by the HMBC correlations from H-12 to C-9, C-13, C-14, and C-16. The HO-12 was assigned as the α-oriented on the basis of the ROESY correlations of H-12/ H-17a (Fig. 3). Therefore, the structure of **3** was determined as 3β-acetoxy-7α,12α,14β-trihydroxy-*ent*-kaur-16-en-15-one.

The ^{1}H and ^{13}C NMR data of pharicinin C (**4**)[14] were similar to those of leukamenin E[15], and the only difference was that an acetoxy was located at C-19 in **4**, which was proven by the HMBC correlations from H_2-19 (δ_H 4.41 and 4.16) to OAc (δ_C 170.9). The relative configuration of **4** was identical to that of leukamenin E according to the ROESY correlations. Consequently, **4** was characterized as 3β,19-diacetoxy-7α,14β-dihydroxy-*ent*-kaur-16-en-15-one.

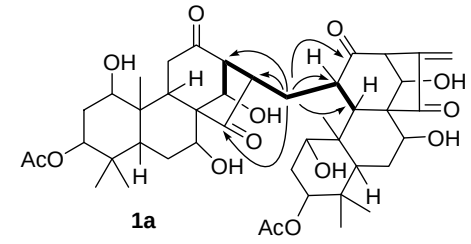
Bispseurata F (**1**) was the first example of dimeric *ent*-kaurane diterpenoid connected by direct linkage of C-17 with C-11′. To clarify whether it is possible to produce **1** from pseurata F in the course of extraction or isolation by the Michael addition dimerization, we carried out some additional experiments, that is, mixed compound pseurata F with silica gel in different solutions (petroleum ether, $CHCl_3$, EtOAc, Me_2CO, and CH_3OH), kept the samples under air at room temperature for one month, and then monitored by the HPLC with **1** and pseurata F as controls. However, **1** was not detected in all mixtures. This showed that bispseurata F (**1**) is a naturally occurring product. A plausible biosynthetic pathway for **1** is proposed, as shown in Scheme 1. A Michael addition reaction is proposed to be the key step of this way. The dimerization of this type of diterpenoid dimer is worth further studies.

Compounds **1**–**4** were evaluated for their cytotoxicity against the NB4 (acute promyelocytic leukemia), A549 (lung cancer), PC-3 (prostate cancer), MCF-7 (breast cancer), and SH-SY5Y (neuroblastoma) human cell lines, using the sulforhodamine B (SRB)

Table 1
^{1}H and ^{13}C NMR data for compounds **1**–**4**[d] [**1** in $(CD_3)_2CO$, **2**–**4** in C_5D_5N, δ in ppm]

No.	**1a**[b]		No.	**1b**[b]		No.	**2**[b]		No.	**3**[c]		No.	**4**[b]	
	^{1}H	^{13}C		^{1}H	^{13}C		^{1}H	^{13}C		^{1}H	^{13}C		^{1}H	^{13}C
1β	3.56 (m)	76.2	1′β	3.80 (m)	75.6	1α 1β	1.75[a] 0.96[a]	39.7	1α 1β	1.42 (m) 1.10 (m)	33.5	1α 1β	1.27[a] 1.04[a]	33.5
2	1.94–1.86[a] (2H)	29.0	2′	1.76-1.67 [a] (2H)	28.8	2α 2β	1.48[a] 0.95[a]	18.4	2α 2β	1.88[a] 1.60[a]	23.1	2α 2β	1.95[a] 1.67[a]	23.1
3α	4.62 (br s)	78.4	3′α	4.66 (br s)	78.4	3α 3β	1.37[a] 1.42[a]	36.5	3α	4.80 (s)	77.7	3α	5.23 (s)	73.0
4		37.4	4′		37.2	4		35.7	4		36.9	4		41.0
5β	1.45 (d, J = 11.9 Hz)	46.4	5′β	1.53[a]	46.7	5β	1.45[a]	44.7	5β	1.60[a]	47.7	5β	1.72[a]	48.3
6α	1.92[a]	33.9	6′α	2.01[a]	34.2	6	2.00[a] (2H)	23.6	6α	1.26 (m)	29.5	6α	2.05[a]	29.4
6β	1.68[a]		6′β	1.76[a]					6β	2.05[a]		6β	2.20 (m)	
7β	4.18 (m)	74.7	7′β	4.38 (m)	74.9	7	6.33 (m)	119.0	7β	4.93 (dd, J = 11.9, 4.4 Hz)	74.5	7β	4.77 (dd, J = 11.8, 4.1 Hz)	74.4
8		61.2	8′		61.4	8		138.7	8		61.7	8		61.8
9β	1.64 (br s)	50.0	9′β	1.55 (br s)	53.0	9β	1.77[a]	51.9	9β	1.88[a]	57.0	9β	1.47[a]	54.7
10		45.0	10′		45.9	10		36.4	10		38.7	10		39.6
11α	3.81 (d, J = 17.1 Hz)	38.6	11′α	4.05 (m)	48.9	11	1.48–1.38[a] (2H)	20.4	11α	1.89[a]	26.6	11α	1.48[a]	17.8
11β	1.70[a]								11β	1.76 (m)		11β	1.28[a]	
12		209.8	12′		209.0	12	1.57–1.49[a] (2H)	35.5	12	4.37 (t, J = 4.3 Hz)	72.4	12α 12β	1.64[a] 1.93[a]	31.3
13α	2.99 (br d, J = 6.9 Hz)	60.4	13′α	3.58 (br s)	64.8	13		43.1	13α	3.59 (d, J = 2.7 Hz)	55.7	13α	3.23 (br s)	46.9
14α	5.01 (br s)	73.2	14′α	5.02 (br s)	74.2	14β	4.02 (br s)	77.3	14α	5.83 (s)	71.2	14α	5.08 (s)	75.3
15		218.7	15′		205.8	15	6.30 (dd, J = 16.3, 10.8 Hz)	149.6	15		208.9	15		207.6
16	3.20 (m)	50.3	16′α		143.8	16a 16b	5.19 (d, J = 16.3 Hz) 5.10 (d, J = 10.8 Hz)	111.4	16		147.7	16		149.6
17a	1.92[a]	35.7	17′a	6.05 (s)	120.7	17	1.13 (s)	15.9	17a	6.31 (s)	117.2	17a	6.31 (s)	116.5
17b	1.12 (m)		17′b	5.53 (s)				37.4	17b	5.41 (s)		17b	5.38 (s)	
18	0.86 (s)	28.0	18′	0.90 (s)	28.0	18a 18b	3.92 (d, J = 10.8 Hz) 3.80 (d, J = 10.8 Hz)	73.3	18	0.86 (s)	28.1	18	1.04 (s)	22.3
19	0.92 (s)	21.8	19′	0.97 (s)	22.0	19	0.91 (s)	18.2	19	0.89 (s)	21.8	19a 19b	4.41 (d, J = 11.4 Hz) 4.16 (d, J = 11.4 Hz)	66.6
20	0.94 (s)	13.6	20′	1.04 (s)	14.0	20	0.89 (s)	16.1	20	1.60 (s)	16.2	20	1.03 (s)	18.0
OAc		170.5	OAc		170.4	OAc		170.9	OAc		170.3	OAc		170.9
	2.01 (s)	21.0		2.01 (s)	21.0		2.00 (s)	20.9		2.05 (s)	21.1		2.07 (s)	21.0
												OAc		170.3
													1.98 (s)	20.6

[a] Signals overlapped.
[b] ^{1}H and ^{13}C NMR spectra were acquired at 400 and 100 MHz, respectively.
[c] ^{1}H and ^{13}C NMR spectra were acquired at 500 and 125 MHz, respectively.
[d] TMS was used as internal standard; assignments were based on HSQC, HMBC, and ROESY spectra.



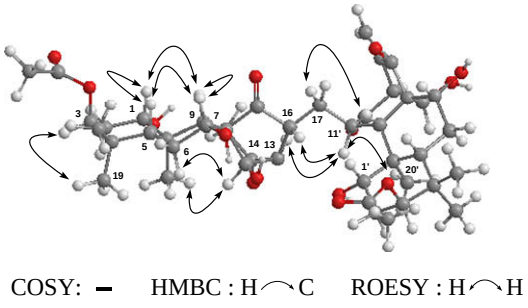


Figure 1. Selected COSY, HMBC, and ROESY correlations of **1**.

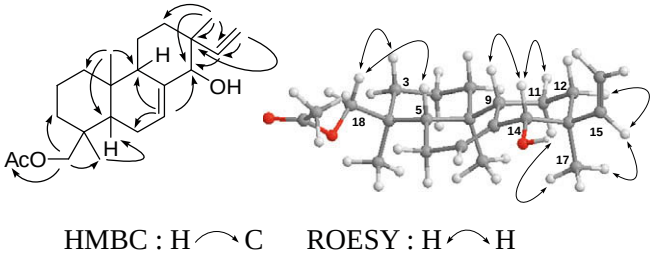


Figure 2. Key HMBC and ROESY correlations of **2**.

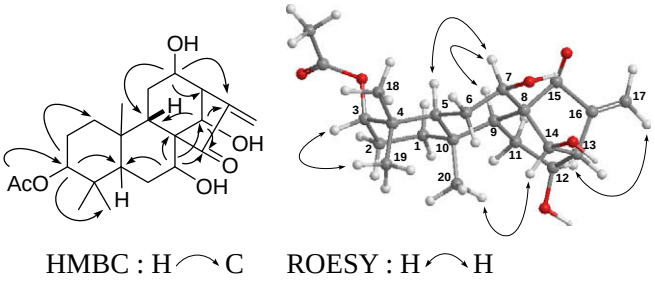
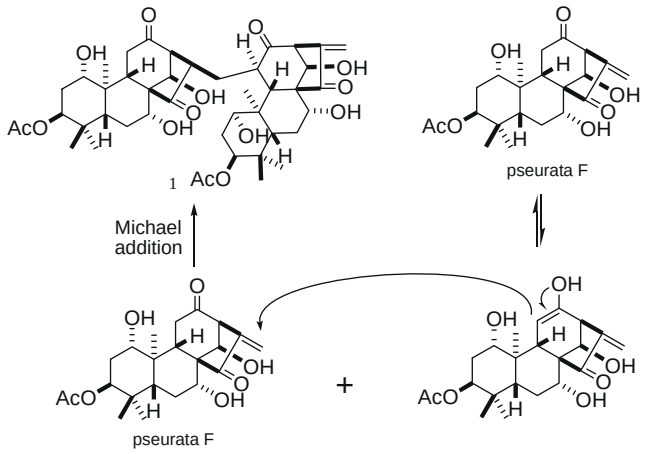


Figure 3. Key HMBC and ROESY correlations of **3**.



Scheme 1. Biogenetic pathway proposed for compound **1**.

Table 2
Cytotoxicity data for compounds **3** and **4** isolated from *I. pharicus* in selected human cell lines[a,b]

Compd	NB4	A549	SH-SY5Y	PC-3	MCF-7
3	3.0	>10	8.1	>10	4.4
4	2.3	>10	5.3	>10	>10
Paclitaxel	0.1	0.1	0.2	0.2	0.1
Etoposide	1.3	1.7	1.7	13.6	7.6

[a] Results are expressed as IC_{50} values in μM.
[b] Compounds **1** and **2** were inactive for all cell lines (IC_{50} > 10 μM).

method, as reported previously,[16] with paclitaxel and etoposide as the positive controls. Among them, compounds **3** and **4** exhibited moderate activities against NB4 cell lines, with values of 3.01 and 2.32 μM, respectively, and against SH-SY5Y cell lines, with values of 8.07 and 5.27 μM, respectively. Compound **3** also exhibited remarkable activities against MCF7 cell lines, with value of 4.36 μM. The results are shown in Table 2.

Acknowledgments

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Supplementary data

Supplementary data (experimental details and ESIMS, 1D and 2D NMR spectra of diterpenoids **1**–**4**) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.02.102.

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9. Bispseurata F (**1**): white, amorphous powder; mp 273–275 °C; $[\alpha]_D^{23}$ +39.1 (*c* 0.01, MeOH); UV (MeOH) λ_{max} (log ε): 215 (3.49) nm; IR (KBr) ν_{max} 3430, 2967, 2881, 1731, 1643, 1466, 1444, 1394, 1376, 1254, 1204, 1181, 1150, 1091, 1077, 1039, 992, 950, 901, 874, 850, 807, 769, 667, 609, 601, 508 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; positive ESIMS *m/z* 835 $[M+Na]^+$; positive HRESIMS $[M+Na]^+$ *m/z* 835.3900 (calcd for $C_{44}H_{60}O_{14}Na$ $[M+Na]^+$, 835.3880).
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11. Pharicinin A (**2**): white powder; mp 91–93 °C; $[\alpha]_D^{19}$ +22.7 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε): 204 (3.52) nm; IR (KBr) ν_{max} 3504, 3075, 2955, 2936, 2921, 2850, 1720, 1639, 1440, 1392, 1380, 1360, 1273, 1166, 1078, 1043, 1013, 1003, 952, 903, 872, 854, 822, 748, 662, 633, 601, 516 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; positive ESIMS *m/z* 369 $[M+Na]^+$; positive HRESIMS $[M+Na]^+$ *m/z* 369.2404 (calcd for $C_{22}H_{34}O_3Na$ $[M+Na]^+$, 369.2405).
12. Pharicinin B (**3**): white powder; mp 149–151 °C; $[\alpha]_D^{20}$ +1.4 (*c* 0.24, MeOH); UV (MeOH) λ_{max} (log ε): 230 (3.42) nm; IR (KBr) ν_{max} 3431, 2933, 2874, 1728, 1649, 1439, 1376, 1252, 1181, 1084, 1028, 988, 933, 843, 813, 745, 711, 675, 637, 603, 535 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; positive ESIMS *m/z* 415 $[M+Na]^+$; positive HRESIMS $[M+Na]^+$ *m/z* 415.2089 (calcd for $C_{22}H_{32}O_6Na$ $[M+Na]^+$, 415.2096).
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14. Pharicinin C (**4**): white powder; $[\alpha]_D^{20}$ −35.3 (*c* 0.47, MeOH); UV (MeOH) λ_{max} (log ε): 230 (3.45) nm; IR (KBr) ν_{max} 3428, 2922, 2873, 2852, 1735, 1649, 1453, 1377, 1249, 1181, 1088, 1062, 1035, 987, 843, 797, 607, 536 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; positive ESIMS *m*/*z* 457 $[M+Na]^+$; positive HRESIMS $[M+Na]^+$ *m*/*z* 457.2220 (calcd for $C_{24}H_{34}O_7Na$ $[M+Na]^+$, 457.2202).
15. Takeda, Y.; Fujita, T.; Ueno, A. *Chem. Lett.* **1981**, 1229–1232.
16. Monks, A.; Scudiero, D.; Skehan, P.; Shoemaker, R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A. *J. Natl. Cancer Inst.* **1991**, *83*, 757–766. NB4 = acute promyelocytic leukemia; A549 = lung cancer; PC-3 = prostate cancer; MCF-7 = breast cancer; SH-SY5Y = neuroblastoma human cell lines.