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MINOR DAPHNANE-TYPE DITERPENOIDS FROM *WIKSTROEMIA RETUSA*

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Key Word Index—*Wikstroemia retusa*; Thymelaeaceae; daphnane diterpenoids; wikstroelides.

Abstract—In addition to huratoxin, pimelea factor P_2, and wikstroelides A–G from the fresh bark, eight daphnane-type diterpenoids, wikstroelides H–O, were isolated from the bark and stem, and their structures established. Cytotoxicity was assayed on some wikstroelides. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

In a preceding paper, we described the isolation and characterization of nine daphnane-type diterpenoids, including wikstroelides A–G (**2**, **3**, **5**–**9**) [1, 2], as well as huratoxin (**1**) [1–4] and pimelea factor P_2 (**4**) [2, 5], from the fresh bark of *Wikstroemia retusa* from the Ryukyu Islands. This paper deals with the isolation of minor daphnane diterpenoids from the fresh bark and the dried bark with stem.

RESULTS AND DISCUSSION

From the fresh bark, four diterpenoids (wikstroelides H–K) (**10**–**13**) were obtained, in addition to the already reported daphnanes, **1**–**9**, and the structures established. The molecular formula of **10** (wikstroelide H) was assigned as $C_{34}H_{46}O_{10}$. The 20-carbinol group appeared to be free, since H-20 was observed at the normal chemical shift at δ 3.80 and 3.94. Signals due to the daphnane skeleton, including 12-*O*-acetyl group, were assignable in the ^1H and ^{13}C NMR spectra, as observed in daphnanes **2**, **3**, **5** and **6**. An unsaturated fatty acid with the *ortho*-ester linkage at 9-, 13- and 14-hydroxyl groups was identified as (2′*E*,4′*E*)-dodeca-2′,4′-dienoic acid, based on a base peak at *m/z* 195 in the negative FAB mass spectrum, as well as chemical shifts and coupling constants of the olefinic protons.

Based on FAB mass spectroscopy, **11** (wikstroelide I) was considered to have the molecular formula, $C_{53}H_{82}O_{11}$. As in **10**, the presence of a 12-*O*-acetyl group in the daphnane skeleton with the *ortho*-ester-type unsaturated acid was observed in the ^1H NMR spectrum. The 20-carbinyl protons were shifted downfield (δ 3.89 and 4.82), showing that a fatty acid was linked to the 20-hydroxyl; the acid was identified as palmitic based on a fragment peak at *m/z* 255 in the negative FAB mass spectrum. The dienoic acid in the *ortho*-ester position was identified as (2′*E*,4′*E*)-pentadeca-2,4-dienoic acid, based on the fragment peak at *m/z* 237 and large coupling constants due to the olefinic protons (16 Hz for H-2′/H-3′; 11 Hz for H-3′/H-4′, 15 Hz for H-4′/H-5′), as was also observed in **10**. Thus, **11** was characterized to be a compound, with one additional carbon in the dienoic acid moiety of wikstroelide D (**6**).

Compound **12** (wikstroelide J) afforded a quasi-$[M]^+$ at *m/z* 683, suggesting it to be 18 mu greater than wikstroelide A (**2**). The presence of the unsaturated acid having a diene system linked as in **2**, **10**, and **11** was shown, besides the daphnane skeleton, in the ^1H, ^{13}C NMR and ^1H–^1H COSY spectra. Two ester carbonyl carbon signals were observed at δ 170.1 and 166.9. The former ones was assignable to that of the acetyl group, the latter to a 2′,4′-dienoic acid. Since the UV absorption maximum was shifted from 230 nm in **11** to 261 nm, the unsaturated acid moiety seemed to be linked not as an *ortho*-ester but as a single ester, and was identified as tetradecadienoic acid based on the molecular formula. The location of the ester linkage was confirmed to be the 14-hydroxyl group by the three-bond correlation between H-14 and the carbonyl carbon of the acid. The stereochemistry of the diene system was assigned to be 2′*E*,4′*E*, since a large coupling constant (15 Hz) between H-2′ and H-3′ was observed and C-6′ was at the lower field (δ 33.1); signals for H-4′ and H-5′,

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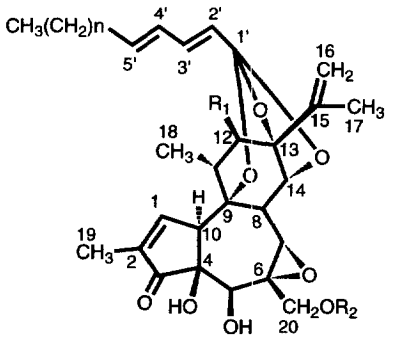
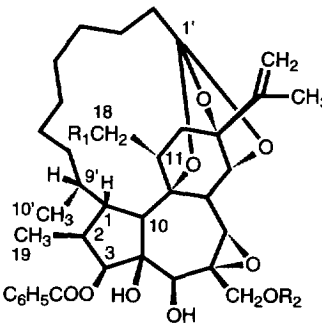
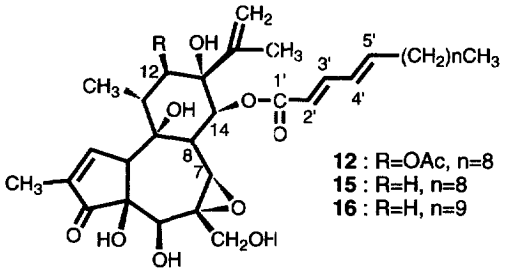


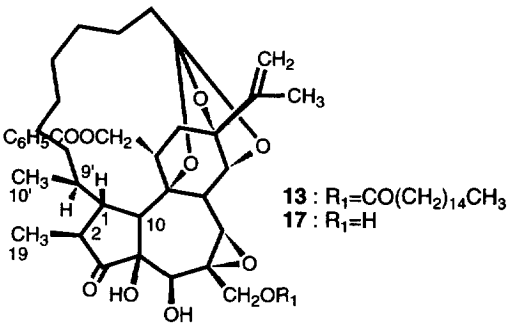
1 : R_1=R_2=H, n=8
2 : R_1=OAc, R_2=H, n=8
3 : R_1=OAc, R_2=H, n=9
5 : R_1=OAc, R_2=*trans*-5-pentadecenoic acid, n=8
6 : R_1=OAc, R_2=$CO(CH_2)_{14}CH_3$, n=8
10 : R_1=OAc, R_2=H, n=6
11 : R_1=OAc, R_2=$CO(CH_2)_{14}CH_3$, n=9
14 : R_1=OAc, R_2=H, n=8, (2′*E*,4′*Z*)



4 : R_1=R_2=H
7 : R_1=R_2=H, 3 : oxo
8 : R_1=C_6H_5COO, R_2=H, 3 : oxo
9 : R_1=C_6H_5COO,
R_2=$CO(CH_2)_{14}CH_3$, 3 : oxo



12 : R=OAc, n=8
15 : R=H, n=8
16 : R=H, n=9



13 : R_1=$CO(CH_2)_{14}CH_3$
17 : R_1=H

however, were not identified. Consequently, **12** was considered to be produced from wikstroelide A (**2**) by cleavage of the *ortho*-ester linkage at C-9 and C-13. While ring C in **2** retained the boat conformation, NOE responses were observed between H-12/H-17, 18 and H-14/H-7, 8, 16, 17, suggesting that ring C in **12** also retains the boat form, as in **2**.

Compound **13** (wikstroelide K) had the same molecular formula, $C_{53}H_{78}O_{11}$, as wikstroelide G (**9**) and the presence of one benzoic and one palmitic acid residue was observed, as in **9**. Although the HMBC spectrum suggested that the acid residues were linked to 18- and 20-hydroxyl groups, respectively, the ^{13}C chemical shifts on C-9′, C-10′ and C-19 were different from those of **9**. As a result of irradiation of H-1, 9′, and 10′ in differential NOE measurements **13** was confirmed to be an isomer of **9** at C-9′, and characterized to retain 9′(*S*)-configuration in the macrocyclic linkage.

Compound **14** (wikstroelide L) was obtained along with **2**–**4**, **7**, **12**, **15**, **16**, and **17** from the dried bark with stem stored at room temperature for six months. When comparing the ^1H and ^{13}C NMR spectra, most signals and coupling constants were similar to those of **2** and the same molecular formula, $C_{36}H_{50}O_{10}$, was observed by FAB mass spectroscopy. The coupling constants between H-4′ and H-5′ in the unsaturated acid moiety were observed as 11 Hz, smaller than 15 Hz in that of **10** or **11**, and C-6′ was at δ 27.9, at higher field than that of **2** at δ 32.7. Compound **14** was characterized to be an isomer of **2**, in which the unsaturated fatty acid in the *ortho*-ester form was (2′*E*,4′*Z*)-tetradecadienoic acid.

Compounds **15** (wikstroelide M) and **16** (wikstroelide N) showed similar signals in their ^1H and ^{13}C NMR spectra, which were also similar to **12** except for an acetoxyl residue. Molecular formulae were suggested to be $C_{34}H_{50}O_9$ and $C_{35}H_{52}O_9$, respectively, by FAB mass spectroscopy. As shown in **12**, the presence of an ester-carbonyl carbon in **15** and **16**, observed at δ 167, suggested that the two compounds had a simple ester linkage of the dienoic acid, which was assignable to be a tetradecadienoic acid and a pentadecadienoic acid, respectively. Therefore, **15** was an ester-derivative corresponding to huratoxin (**1**). The stereochemistry of the diene structure was assigned as 2′*E* and 4′*E*, based on the coupling constants.

The NMR spectra of **17** (wikstroelide O) were similar to those of **13**, but showed no signals for palmitic acid. Absence of a hydroxyl group at C-12 was confirmed by the methylene proton signals at δ 2.05 (*dd*, J = 14.7 Hz) and 2.33 (*d*, J = 14 Hz) assignable to H-12, and a quasi-$[M]^+$ at *mz* 675 ($C_{37}H_{48}O_{10}$+Na). As in **13**, the stereochemistry at C-9′ was also assigned as *S* by comparison between the ^{13}C NMR signals of C-9′, C-10′ and C-19 with those of **13** and wikstroelide F (9′*R*) (**8**).

Cytotoxicity assay was carried out on some of the major wikstroelides, A (**2**), C (**5**), E (**7**), I (**11**), J (**12**) and pimelea factor P_2 (**4**), using cell lines PC-6 and

Table 1. ^{1}H NMR spectral data for compounds **10–16** ad **17** [δ in $CDCl_3$ (500 MHz)]

H	10	11	12	13	14	15	16	17
1	7.56 (*br s*)	7.55 (*br s*)	7.66 (*br s*)	2.50 (*t*, 12)	7.55 (*br s*)	7.66 (*br s*)	7.66 (*br s*)	2.52 (*t*, 12)
2				2.35 (m)				2.35 (*m*)
5	4.25 (*s*)	4.26 (*s*)	4.28 (*s*)	4.03 (*s*)	4.26 (*d*, 2)	4.25 (*s*)	4.25 (*s*)	4.09 (3)
7	3.49 (*s*)	3.42 (*s*)	3.25 (*s*)	3.32 (*s*)	3.55 (*br s*)	3.17 (*s*)	3.17 (*s*)	3.42 (*s*)
8	3.50 (*d*, 2)	3.50 (*d*, 2)	4.10 (*d*, 5)	3.01 (*d*, 3)	3.50 (*br s*)	3.65 (*br s*)	3.65 (*d*, 2)	3.02 (*d*, 3)
10	3.83 (*br s*)	3.87 (*br s*)	3.58 (*t*, 2)	3.26 *d*, 10)	3.83 (*t*, 3)	3.87 (*br s*)	3.86 (*br s*)	3.25 (*d*, 10)
11			2.30 (*qd*, 7, 3)	2.80 (*dd*, 8, 7)	2.38 (*q*, 7)	2.11 (*m*)		2.78 (*br t*, 8)
12	4.98 (*br s*)	4.99 (*s*)	4.89 (*d*, 3)	2.05 (*dd*, 15, 7)	4.99 (*s*)	1.8–1.9 (*m*)		2.05 (*dd*, 14, 7)
				2.36 (*d*, 15)				2.33 (*d*, 14)
14	4.75 (*d*, 2)	4.71 (*d*, 2)	5.91 (*d*, 5)	4.31 (*d*, 3)	4.76 (*d*, 2)	5.67 (*br s*)	5.67 (*d*, 1)	4.33 (*d*, 3)
16	4.96 (*br s*)	4.96 (*br s*)	5.04 (*br s*)	4.92 (*br s*)	4.97 (*br s*)	5.07 (*br s*)	5.07 (*br s*)	4.93 (*br s*)
	5.01 (*br s*)	5.01 (*br s*)	5.24 (*br s*)	5.04 (*br s*)	5.02 (*br s*)	5.13 (*br s*)	5.13 (*br s*)	5.04 (*br s*)
17	1.83 (*br s*)	1.83 (*br s*)	1.86 (*br s*)	1.80 (*br s*)	1.84 (*br s*)	1.88 (*d*, 1)	1.89 (*br s*)	1.80 (*br s*)
18	1.29 (*d*, 7)	1.30 (*d* 6)	1.29 (*d*, 7)	4.57 (*dd*, 11, 8)	1.30 (*d*, 7)	1.05 (*d*, 6)	1.05 (*d*, 6)	4.58 (*dd*, 10, 9)
				4.82 (*d*, 11)				4.82 (*br d*, 10)
19	1.79 (*br s*)	1.79 (*d*, 1)	1.81 (*br s*)	1.22 (*d*, 7)	1.79 (*d*, 1)	1.79 (*d*, 1)	1.78 (*br s*)	1.22 (*d*, 7)
20	3.80 (*d*, 13)	3.89 (*d* 12)	3.66 (*d*, 12)	3.80 (*d*, 12)	3.82 (*dd*, 12, 6)	3.69 (*d*, 12)	3.70 (*d*, 12)	3.78 (*br d*, 12)
	3.94 (*d*, 13)	4.82 (*d*, 12)	3.91 (*d*, 12)	4.76 (*d*, 12)	3.94 (*dd*, 12, 6)	3.86 (*d*, 12)	3.85 (*d*, 12)	3.86 (*br d*, 12)
Others	5.64 (*d*, 16)	5.63 (*d*, 16)	5.88 (*d*, 15)	2.43 (*m*)	5.72 (*d*, 15)	5.90 (*d*, 15)	5.90 (*d*, 15)	2.43 (*m*)
	(H-2′)	(H-2′)	(H-2′)	(H-9′)	(H-2′)	(H-2′)	(H-2′)	(H-9′)
	6.65 (*dd*, 16, 11)	6.65 (*dd*, 16, 11)	7.33 (*dd*, 15, 10)	1.06 (*d*, 6)	6.97 (*dd*, 15, 11)	7.33 (*dd*, 15, 10)	7.33 (*dd*, 15, 10)	1.06 (*d*, 6)
	(H-3′)	(H-3′)	(H-3′)	(H-10′)	(H-3′)	(H-3′)	(H-3′)	(H-10′)
	6.04 (*dd*, 15, 11)	6.03 (*dd*, 15, 11)	6.10–6.20 (2H)	8.04 (*dd*, 9, 1)	6.00 (*t*, 11)	6.10–6.20 (2H)	6.12–6.22 (2H)	8.04 (*dd*, 9, 1)
	(H-4′)	(H-4′)	(H-4′,5′)	(H-2″,6″)	(H-4′)	(H-4′,5′)	(H-4′,5′)	(H-2″,6″)
	5.85 (*dt*, 15, 7)	5.84 (*dt*, 15, 7)	0.88 (*t*, 7)	7.43 (*t*, 9)	5.60 (*dt*, 11, 7)	0.88 (*t*, 7)	0.88 (*t*, 7)	7.43 (*t*, 9)
	(H-5′)	(H-5′)	(H-14′)	(H-3″,5″)	(H-5′)	(H-14′)	(H-15′)	(H-3″,5″)
	0.88 (*t*, 7)	0.88 (*t*, 7)		7.55 (*t*, 9)	0.88 (*t*, 7)			7.55 (*t*, 9)
	(H-12′)	(H-15′)		(H-4″)	(H-14′)			(H-4″)
	1.98 (*s*, OAc)	1.99 (*s*, OAc)	1.99 (*s*, OAc)		3.52 (*d*, 2)			3.23 (*d*, 3)
					(5-OH)			(5-OH)

Coupling constants (J in Hz) given in parentheses.

Table 2. ^{13}C NMR spectral data for compounds **10**, **12**–**16** and **17** [δ in $CDCl_3$ (125 MHz)]

C	10	12	13	14	15	16	17
1	160.4	160.0	48.6	160.3	162.3	162.4	48.7
2	136.9	137.2	43.4	137.0	134.8	134.8	43.4
3	209.5	209.2	220.0	209.5	209.4	209.5	220.5
4	72.2	72.4	75.6	72.3	72.4	72.3	75.6
5	72.1	70.9	69.7	72.1	70.9	71.2	71.5
6	60.5	62.1	59.4	60.5	61.9	61.8	60.6
7	64.3	62.5	63.8	64.3	63.6	63.7	64.0
8	35.5	39.9	36.3	35.5	39.3	39.3	36.4
9	78.2	75.1	80.3	78.2	76.7	76.4	80.3
10	47.5	50.8	44.1	47.5	49.9	50.0	44.2
11	44.1	42.7	41.5	44.1	37.5	37.5	41.5
12	80.5	79.3	30.4	78.3	37.8	37.9	30.5
13	83.7	75.6	84.1	83.8	74.1	74.1	84.1
14	80.5	72.7	81.9	80.6	77.8	77.9	82.0
15	143.2	144.7	146.1	143.2	145.9	145.9	146.1
16	113.3	114.8	111.4	113.3	114.0	114.0	111.4
17	18.7	19.6	18.9	18.7	18.9	18.9	18.8
18	18.2	16.1	68.2	18.3	18.2	18.2	68.3
19	9.8	9.9	14.6	9.9	9.8	9.8	14.7
20	65.1	65.6	66.3	65.1	65.2	65.2	65.4
1′	117.1	166.9	120.1	117.1	167.4	167.1	120.1
Others	122.3 (2′)	117.9 (2′)	30.0 (9′)	124.4 (2′)	117.8 (2′)	117.8 (2′)	30.2 (9′)
	135.1 (3′)	147.1 (3′)	18.9 (10′)	129.7 (3′)	147.3 (3′)	147.3 (3′)	18.9 (10′)
	128.6 (4′)	128.2 (4′)	130.5 (1″)	126.9 (4′)	128.2 (4′)	128.2 (4′)	130.5 (1″)
	139.3 (5′)	146.4 (5′)	129.6 (2″,6″)	136.4 (5′)	146.6 (5′)	146.6 (5′)	129.6 (2″,6″)
	32.7 (6′)	33.1 (6′)	128.4 (3″,5″)	27.9 (6′)	33.1 (6′)	33.1 (6′)	128.4 (3″,5″)
	14.1 (12′)	14.1 (14′)	132.9 (4″)	14.1 (14′)	14.1 (14′)	14.1 (15′)	132.9 (4′)
	21.1 (OAc)	21.1 (OAc)	166.5 (7″)	21.1 (OAc)			166.6 (7″)
	169.9	170.1	173.6 (1‴)	169.6			
			14.1 (16‴)				

P388, by the MTT assay procedure [6,7]. Although no remarkable activity was observed to PC-6 (human lung cancer cell line), some of them were active when P388 (mouse leukaemia cell line) was used. Of all samples, the macrocyclic type, e.g. **4** and **7**, exhibited the highest activity, followed by **2** and **12**, those having the *ortho*-ester group without a fatty acid at the 20-hydroxyl. Compounds **6** and **11**, the *ortho*-ester compounds with palmitic acid at 20-OH, showed the weakest activity (Table 3).

Wikstroelide E (**7**), the major daphnane diterpenoid in the fresh bark, was obtained in lower yield from the dried bark with stem. While **15** was the dominant constituent of those found in the dried material, no **1** was obtained possibly due to the cleavage of the *ortho*-ester linkage during storage.

EXPERIMENTAL

General. ^1H NMR: 400 or 500 MHz. ^{13}C NMR: 100 MHz. $CDCl_3$, TMS as int. standard. UV: MeOH.

Plant material. Origin of fr. bark of *W. retusa* A. Gray is described in ref. [1]. The bark with stem was collected in Okinawa Island in September, 1995 and dried at room temp. for 6 months (Voucher, FUK 950906S).

Extraction and isolation. Procedures for extraction and isolation from fr. bark (8 kg) are described in ref. [1] and compounds **10**–**13** were obtained following **1**–**9**; **10** (4 mg), **11** (7 mg), **12** (13 mg) and **13** (2 mg). From dried bark with stem (36 kg), the following compounds were obtained. **2** (12 mg), **3** (11 mg), **4**, (8 mg), **7** (11 mg), **12** (10 mg), **14** (6 mg), **15** (80 mg), **16** (11 mg) and **17** (5 mg).

Compound **10** (*wikstroelide H*). Solid. $[\alpha]_D^{24}$ +25.3° (MeOH, *c* 0.08). UV λ_{max} nm (log ε): 230 (4.51). FAB MS *m/z*: 615.3173. $C_{34}H_{46}O_{10}+H$ requires 615.3170. Negative FAB MS *m/z*: 195 ($C_{12}H_{19}O_2$, base). ^1H and ^{13}C NMR: Tables 1 and 2.

Compound **11** (*wikstroelide I*). Solid. $[\alpha]_D^{24}$ +60.0° (MeOH, *c* 0.06). UV λ_{max} nm (log ε): 230 (sh) (4.40). FAB MS *m/z*: 895.5930. $C_{53}H_{82}O_{11}+H$ requires 895.5936. Negative FAB MS *m/z*: 255, 237. ^1H NMR: Table 1.

Compound **12** (*wikstroelide J*). Solid. $[\alpha]_D^{32}$ +8.6° (MeOH, *c* 0.67). UV λ_{max} nm (log ε): 261 (4.30). FAB MS *m/z*: 683.3406. $C_{36}H_{52}O_{11}+Na$ requires 683.3407. Negative FAB MS *m/z*: 447, 223, 59. ^1H and ^{13}C NMR. Tables 1 and 2. Cross-peaks in HMBC spectrum: H-14/C-1′, 7, 9, 15; H-12/C-14, 15, 18, -$COCH_3$; H-16/C-13, 17. NOESY: H-14/H-7, 8, 16, 17; H-12/H-18, 17.

Compound **13** (*wikstroelide K*). Solid. $[\alpha]_D^{23}$ +50.0° (MeOH, *c* 0.08). UV λ_{max} nm (log ε): 228 (4.12). FAB

Table 3. Cytotoxic activity of some wikstroelides [GI_{50} (ng ml^{-1})]

Compound	P388‡	PC-6§
4	0.27	7090
7	0.77	11800
2	2.49	8110
12	4.62	21800
6	40.4	39900
11	48.3	16500
CDDP*	14.8	235
VCR†	0.47	0.26

* CDDP = cisplatin.
† VCR = vincristine sulphate.
‡ Mouse leukaemia cell line.
§ Human lung cancer cell line.

MS *m/z*: 913.5439. $C_{53}H_{78}O_{11}$ + Na requires 913.5442. ^1H and ^{13}C NMR: Tables 1 and 2. Cross-peaks in HMBC spectrum: H-18/C=O in benzoic acid; H-20/C=O in palmitic acid. Differential NOE: H-1/H-10′, 11, 18; H-9′/H-10, 10′, 18; H-10′/H-1.

Compound **14** (*wikstroelide L*). Solid. $[\alpha]_D^{33}$ +15.0° (MeOH, *c* 0.26). UV λ_{max} nm (log ε): 232 (4.24). FAB MS *m/z*: 643.3477. $C_{36}H_{50}O_{10}$ + H requires 643.3483. ^1H and ^{13}C NMR: Tables 1 and 2.

Compound **15** (*wikstroelide M*). Solid. $[\alpha]_D^{32}$ +9.1° (MeOH, *c* 0.79). UV λ_{max} nm (log ε): 260 (4.25). FAB MS *m/z*: 625.3360. $C_{34}H_{50}O_9$ + Na requires 625.3353. ^1H and ^{13}C NMR: Tables 1 and 2.

Compound **16** (*wikstroelide N*). Solid. $[\alpha]_D^{33}$ +18.1° (MeOH, *c* 0.32). UV λ_{max} nm (log ε): 260 (4.41). FAB MS *m/z*: 639.3505. $C_{35}H_{52}O_9$ + Na requires 639.3509. ^1H and ^{13}C NMR: Tables 1 and 2.

Compound **17** (*wikstroelide O*). Solid. $[\alpha]_D^{32}$ +78.3° (MeOH, *c* 0.12). UV λ_{max} nm (log ε): 228 (4.12). FAB MS *m/z*: 675.3148. $C_{37}H_{48}O_{10}$ + Na requires 675.3145. ^1H and ^{13}C NMR: Tables 1 and 2.

Cytotoxicity assay. Cellular growth of PC-6 and P388 in the presence or absence of the samples was determined according to the MTT assay procedure [6, 7]. The activity of each sample is given as GI_{50} (ng ml^{-1}) (Table 3).

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