Antitumor Agents, 145. Cytotoxic Asprellic Acids A and C and Asprellic Acid B, New p-Coumaroyl Triterpenes, from Ilex asprella

Yoshiki Kashiwada, De-Cheng Zhang, Yuh-Pan Chen, Chang-Ming Cheng, Hsien-Tsung Chen, Hsien-Chang Chang, Jer-Jang Chang, and Kuo-Hsuing Lee

J. Nat. Prod., **1993**, 56 (12), 2077-2082• DOI: 10.1021/np50102a008 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 56, No. 12, pp. 2077-2082, December 1993

ANTITUMOR AGENTS, 145.[1] CYTOTOXIC ASPRELLIC ACIDS A AND C AND ASPRELLIC ACID B, NEW *p*-COUMAROYL TRITERPENES, FROM *ILEX ASPRELLA*

YOSHIKI KASHIWADA, DE-CHENG ZHANG,

Natural Products Laboratory, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599

YUH-PAN CHEN, CHANG-MING CHENG, HSIEN-TSUNG CHEN, HSIEN-CHANG CHANG,

Brion Research Institute of Taiwan, Chung-Ching South Road, Sec. 3, Taipei, Taiwan 100, Republic of China

JER-JANG CHANG,
Department of Pathology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599

and KUO-HSUING LEE*

Natural Products Laboratory, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599

ABSTRACT.—Three new *p*-coumaroyl triterpenes, asprellic acids A [**1**], B [**2**], and C [**3**], were isolated from the EtOH extract of dried leaves of *Ilex asprella*. The structures for **1–3** have been established as 3, 27-di-*O-trans-p*-coumaroyl- [**1**], 3-*O-trans-p*-coumaroyl-27-*O-cis-p*-coumaroyl- [**2**], and 3-*O-cis-p*-coumaroyl-27-*O-trans-p*-coumaroyl- [**3**] 3β,27-dihydroxyolean-12-en-28-oic acid, respectively, by spectral and chemical examinations. Asprellic acid A [**1**] exhibited potent cytotoxicity against the RPMI-7951 cell line with an ED_{50} value of 0.62 μg/ml, while the cytotoxicity of asprellic acid C [**3**] against RPMI-7951 was marginal (ED_{50} 5.5 μg/ml). Compounds **1** and **3** also showed cytotoxicity against KB cells with ED_{50} values of 3.75 and 2.86 μg/ml, respectively. Asprellic acid B [**2**] did not show cytotoxicity (>10 μg/ml) against KB or RPMI-7951 cell lines.

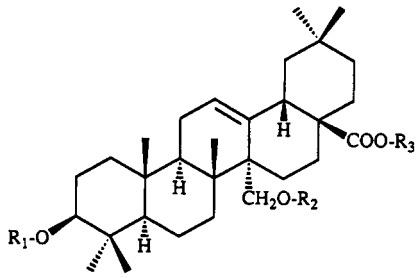
In the course of our continuing search for antitumor agents from plant natural products, the EtOH extract of *Ilex asprella* (Hook. & Arn.) Champ. ex Benth. (Aquifoliaceae) was found to show significant cytotoxicity against KB (epidermoid carcinoma of the nasopharnyx) and RPMI-7951 (melanoma) cell lines. Subsequent bioassay-guided fractionation and column chromatography repeated on Si gel led to the isolation of three new triterpenoids, asprellic acids A–C. We report here on the isolation and characterization of these compounds and on their cytotoxicity.

RESULTS AND DISCUSSION

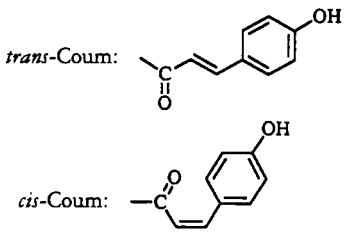
Asprellic acid A [**1**], colorless needles (from EtOAc), mp 250–252°, was positive to a Liebermann-Burchard reaction and gave an $[M-H]^-$ ion peak at *m/z* 763 in the negative fabms. The ^{1}H-nmr spectrum of **1** showed the presence of six tertiary methyls [δ 0.90, 0.95, 0.97, 0.99, 1.01, and 1.07 (each 3H, s)], hydroxy-bearing methylene [δ 4.62, 4.74 (each 1H, d, J=12.5 Hz)] and methine [δ 4.90 (1H, dd, J=5, 11 Hz)] groups, and an olefinic group [δ 5.85 (1H, brs)]. At lower field, two pairs of trans-coupled olefinic [δ 6.69, 6.76, 8.01, and 8.06 (each 1H, d, J=16 Hz)] and A_2B_2-type aromatic [δ 7.18, 7.19, 7.66 and 7.68 (each 2H, d, J=8 Hz)] signals were present, suggesting the existence of two *p*-coumaroyl groups in **1**.

Methylation of **1** with Me and K_2CO_3 gave a trimethylate, which showed an $[M+Na]^+$ ion peak at *m/z* 829 in the fabms. Alkaline hydrolysis of **1** with 5% KOH/

[1]For Part 144, see Zhou *et al.* (1).



1 $R_1=R_2=$*trans*-Coum, $R_3=H$
2 $R_1=$*trans*-Coum, $R_2=$*cis*-Coum, $R_3=H$
3 $R_1=$*cis*-Coum, $R_2=$*trans*-Coum, $R_3=H$
4 $R_1=R_2=R_3=H$
5 $R_1=R_2=H$, $R_3=Me$



MeOH yielded a hydrolysate **4** and a phenolic carboxylic acid; the latter compound was found to be *p*-coumaric acid by direct comparison with an authentic sample. In the negative fabms, **4** gave an $[M-H]^-$ ion peak at *m/z* 471. The ^{13}C-nmr spectrum of **4** showed 30 carbons including six methyls, a trisubstituted double bond, two carbons carrying an oxygen function, and a carboxylic acid, suggesting **4** to be a triterpene. Comparison of the ^{13}C-nmr data of **4** with those of known triterpenes suggested an oleanane-type skeleton. The ^{1}H-nmr spectrum of **4** exhibited, together with six tertiary methyl signals, a hydroxy-carrying methine [δ 3.37 (dd, J=5, 11 Hz)] and olefinic [δ 5.88 (brs)] signals, which were assignable to H-3 and H-12, respectively. It also showed AB-type methylene signals at δ 3.83 and 4.09 (each 1H, d, J=12 Hz), suggesting the presence of a hydroxymethyl group. Examination of the long-range ^{1}H- ^{13}C COSY of **4** indicated that this hydroxymethyl group was located at C-14, based on the observation of long-range correlations between the hydroxymethyl signals and C-13 and C-8. Subsequently, **4** was methylated with CH_2N_2 to yield a monomethylate **5**, which was identified as methyl 3β,27-dihydroxyolean-12-en-28-oate by comparison of its physical and spectral data with those described in the literature (2).

The *p*-coumaroyl groups in **1** were located at the C-3 and C-27 hydroxyl groups, since in the ^{1}H-nmr spectrum of **1**, the signals ascribable to H-3 [δ 4.90 (1H, dd, J=5, 11 Hz)] and H-27 [δ 4.62, 4.74 (each 1H, d, J=12.5 Hz)] were shifted downfield compared with those of **4**. On the basis of the chemical and spectral evidence above, the structure of **1** was established as 3,27-di-*O*-*trans*-*p*-coumaroyl-3β,27-dihydroxyolean-12-en-28-oic acid.

Asprellic acids B [**2**] and C [**3**] both gave an $[M-H]^-$ ion peak at *m/z* 763 in the negative fabms, which was identical with that found in **1**. The ^{1}H nmr of **2** and **3** resembled that of **1**, showing the presence of six tertiary methyl, hydroxy-bearing methine and methylene groups, and an olefinic group in each molecule. At lower field, they also showed signals similar to those of **1**, except for the observation of a pair of olefinic signals with cis coupling [δ 6.05 and 7.07 (each 1H, d, J=13 Hz) in **2**; δ 6.01

and 6.98 (each 1H, d, J=13 Hz) in **3**], suggesting that one of the *p*-coumaroyl groups in **2** and **3** has a cis configuration. Alkaline hydrolysis of **2** and **3** gave the same hydrolysate found for **1**. The location of the *cis-p*-coumaroyl group in **2** was concluded to be at the C-27 hydroxyl group, since in the ^{1}H-nmr spectrum of **2**, the methylene signal assignable to H-27 was observed as a singlet [δ 4.58 (2H, s)], while the signal [δ 4.89 (1H, dd, J=5, 11 Hz)] due to H-3 was similar to that found in **1**. In contrast, in the ^{1}H nmr of **3**, the H-3 signal [δ 4.82 d, J=5, 11 Hz)] was shifted upfield, whereas the signals due to H-27 were observed at δ 4.58 and 4.72 (each 1H, d, J=12.5 Hz). This latter chemical shift and coupling pattern were almost the same as those found in **1**. Accordingly, the location of the *cis-p*-coumaroyl group in **3** was concluded to be at the C-3 hydroxyl group. On the basis of this evidence, the structures of **2** and **3** were concluded to be 3-*O-trans-p*-coumaroyl-27-*O-cis-p*-coumaroyl- [**2**] and 3-*O-cis-p*-coumaroyl-27-*O-trans-p*-coumaroyl- [**3**] 3β,27-dihydroxyolean-12-en-28-oic acid, respectively.

Asprellic acid A [**1**] exhibited potent cytotoxicity against the RPMI-7951 cell line, with an ED_{50} value of 0.62 μg/ml, while the cytotoxicity of asprellic acid C [**3**] against the RPMI-7951 was marginal (ED_{50} 5.5 μg/ml). Compounds **1** and **3** also showed cytotoxicity against KB cells with ED_{50} values of 3.75 and 2.86 μg/ml, respectively. They were inactive (>10 μg/ml) against A-549 (lung carcinoma), HCT-8 (ileocecal adenocarcinoma), and P-388 (leukemia) cell lines. Since asprellic acid B [**2**] did not show cytotoxicity (>10 μg/ml) against KB or RPMI-7951 cell lines, the *trans-p*-coumaroyl group at C-27 might be important to retain cytotoxicity.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All melting points were determined on a Fisher-Johns micro-melting point apparatus and are uncorrected. Optical rotations were measured with an AUTOPOL III automatic polarimeter. Mass spectra were obtained on a JEOL HX-110 spectrometer. ^{1}H- and ^{13}C-nmr

TABLE 1. ^{1}H nmr Data for **1**, **4**, **2**, and **3** (in pyridine-d_5).

Proton	Compound			
	1	**4**	**2**	**3**
H-3	4.92 (dd, J=5, 11 Hz)	3.37 (dd, J=5, 11 Hz)	4.89 (dd, J=5, 11 Hz)	4.82 (dd, J=4.5, 11 Hz)
H-12	5.85 (1H, brs)	5.88 (1H, brs)	5.79 (1H, brs)	5.85 (1H, brs)
H-18	3.42 (dd, J=4, 14 Hz)	3.41 (dd, J=4, 14 Hz)	3.58 (dd, J=4, 14 Hz)	3.42 (dd, J=4, 14 Hz)
H-27	4.62, 4.74 (each d, J=12.5 Hz)	3.83, 4.09 (each d, J=12 Hz)	4.58 (2H, s)	4.58, 4.72 (each d, J=12.5 Hz)
4α-Me	0.97 (s)	1.20 (s)	0.96 (s)	0.93 (s)
4β-Me	0.99 (s)	1.03 (s)	0.99 (s)	0.95 (s)
8-Me	1.07 (s)	1.05 (s)	1.01 (s)	1.05 (s)
10-Me	0.90 (s)	0.91 (s)	0.89 (s)	0.87 (s)
20α-Me	0.95 (s)	0.88 (s)	0.92 (s)	0.90 (s)
20β-Me	1.01 (s)	1.03 (s)	1.00 (s)	1.00 (s)
p-Coumaroyl				
H-2	7.66, 7.68 (each d, J=8 Hz)		7.66, 8.15 (each d, J=8 Hz)	7.66, 8.09 (each d, J=8 Hz)
H-3	7.18, 7.19 (each d, J=8 Hz)		7.24 (2H, d, J=8 Hz)	7.18 (2H, d, J= 8 Hz)
−CH=CH−	6.69, 8.01 (each d, J=16 Hz)		6.05, 7.07 (each d, J=13 Hz)	6.01, 6.98 (each d, J=13 Hz)
	6.76, 8.06 (each d, J=16 Hz)		6.69, 8.00 (each d, J=16 Hz)	6.75, 8.04 (each d, J=16 Hz)

spectra were measured on a Bruker AC 300 spectrometer and are given in ppm (δ) downfield from an internal TMS standard. Cc was carried out with Silica 32–63 (32–63 μ, Universal Adsorbents Inc.). Tlc was performed on Kiesel gel 60 F254 plates (0.2 mm thick, Merck).

PLANT MATERIAL.—The leaves of *I. asprella* used in this investigation were from a collection made in May 1991, at Yang Ming Shan, Taipei, Taiwan. A voucher specimen is kept in the Herbarium of Brion Research Institute of Taiwan, Taipei.

EXTRACTION AND ISOLATION OF ASPRELLIC ACIDS A–C.—Dried leaves of *I. asprella* (6.3 kg) were extracted with EtOH three times.The EtOH extract, after removal of the solvent by evaporation, was subjected to Si gel cc. Elution with C_6H_6-EtOAc (85:15→72:28) gave three fractions. Fraction 2 was further

TABLE 2. ^{13}C-nmr Data for **1**, **4**, **5**, **2**, and **3** (75 MHz).

Carbon	Compound				
	1[a]	**4**[a]	**5**[b]	**2**[a]	**3**[a]
Triterpenoid moiety					
C-1	38.3	38.9	38.1	38.2	38.0
C-2	28.4	28.1	27.1	28.3	28.2
C-3	80.7	78.0	78.6	80.8	80.7
C-4	38.4	39.4	38.7	38.4	38.4
C-5	55.7	55.8	54.8	55.7	55.6
C-6	18.7	18.9	18.2	18.7	18.6
C-7	33.6	33.8	33.0	33.5	33.6
C-8	40.6	40.5	39.7	40.5	40.5
C-9	49.0	48.8	48.3	49.0	48.9
C-10	37.6	37.6	37.0	37.6	37.5
C-11	23.7	23.8	22.6	23.8	23.7
C-12	127.1	127.6	129.4	127.2	127.0
C-13	139.1	139.9	137.9	138.9	139.0
C-14	46.6	48.0	47.5	46.5	46.5
C-15	24.5	24.5	24.5	24.4	24.4
C-16	24.2	24.0	24.1	24.1	24.1
C-17	46.2	46.6	46.3	46.0	46.1
C-18	41.9	41.9	40.7	41.8	41.9
C-19	45.6	45.6	44.9	45.6	45.6
C-20	31.0	31.0	30.8	31.0	30.9
C-21	34.2	34.1	33.5	34.2	34.2
C-22	33.3	33.2	32.2	33.3	33.2
C-23	28.4	28.8	28.0	28.3	28.2
C-24	17.3	16.6	15.7	17.3	17.1
C-25	15.8	16.1	15.5	15.8	15.8
C-26	18.7	18.9	18.2	18.7	18.6
C-27	66.2	64.5	62.9	66.3	66.1
C-28	180.2	180.2	177.9	180.3	180.2
C-29	33.3	33.2	32.5	33.3	33.2
C-30	23.9	23.9	23.9	23.9	23.8
OMe			51.7		
p-Coumaroyl Moiety					
C-1	126.3, 126.4			126.3, 126.7	126.2, 126.7
C-2	130.8, 130.9			130.8, 133.8	130.8, 133.7
C-3	116.8, 116.9			116.1, 116.9	116.0, 117.0
C-4	161.4, 161.5			160.6, 161.4	160.6, 161.6
−CH=CH−	115.7, 115.9			115.9, 116.5	115.6, 116.9
	145.1, 145.4			144.6, 145.1	143.7, 145.3
−COO−	167.3, 167.5			166.8, 167.4	166.6, 167.3

[a]Measured in pyridine-d_5.
[b]Measured in $CDCl_3$.

chromatographed on Si gel with $CHCl_3$-MeOH (40:1→30:1) to yield asprellic acid B [**2**] (81 mg) and a mixture of asprellic acids A [**1**] and C [**3**]. The mixture was separated by Si gel cc with C_6H_6-EtOAc (3:1→2:1) to afford pure samples of **1** (1.38 g) and **3** (52 mg).

Asprellic acid A [**1**].—Colorless needles (from EtOAc): mp 250–252°; $[\alpha]^{20}D$ +122.0° (c=0.85, pyridine); negative fabms m/z $[M-H]^-$ 763; hrfabms m/z $[M+Na]^+$ 787.4191 ($C_{48}H_{60}O_8Na$, calcd 787.4186); ^1H nmr see Table 1; ^{13}C nmr see Table 2.

Methylation of **1**.—A mixture of **1** (150 mg), anhydrous K_2CO_3 (500 mg), and MeI (0.5 ml) in dry Me_2CO (20 ml) was refluxed for 3 h. After removal of the inorganic salts by filtration, the filtrate was concentrated to a solid, which was crystallized from MeOH to furnish the trimethylate (112 mg) as colorless needles: mp 203–204°; $[\alpha]^{20}D$ +156.8° (c=0.56, $CHCl_3$); fabms m/z $[M+Na]^+$ 829, 651, 629, 451, 391; hrfabms m/z $[M+Na]^+$ 829.4651 ($C_{51}H_{66}O_8Na$, calcd 829.4655); ^1H nmr ($CDCl_3$) δ 0.68, 0.77, 0.83, 0.84, 0.86, 0.90 (each 3H, s, 4α, 4β, 8-, 10-, 20α-, and 20β-Me), 2.87 (1H, dd, J=4, 14 Hz, H-18), 3.58 (3H, s, COOMe), 3.76, 3.78 (each 3H, s, OMe), 4.09, 4.27 (each 1H, d, J=12.5 Hz, H-27), 4.51 (brt, J=7.5 Hz, H-3), 5.55 (1H, brs, H-12), 6.18, 6.23 (each 1H, d, J=16 Hz, olefinic-H), 6.82, 6.85 (each 2H, d, J=8 Hz, *p*-coumaroyl H-3), 7.40, 7.42 (each 2H, d, J=8 Hz, *p*-coumaroyl H-2), 7.53 (2H, d, J=16 Hz, olefinic-H); ^{13}C nmr ($CDCl_3$) δ 15.6 (C-25), 16.9 (C-24), 18.0 (C-26), 18.2 (C-6), 22.8 (C-11), 23.6 (C-30), 23.5, 24.0 (C-15, -16), 28.1 (C-2, -23), 30.6 (C-20), 32.3, 32.9 33.7 (C-7, -21, -22, -29), 37.1 (C-10), 37.9 (C-1), , 38.1 (C-4), 40.0 (C-8), 41.1 (C-18), 44.7 (C-19), 45.2 (C-17), 46.5 (C-14, 48.5 (OMe), 51.6 (C-9), 55.2, 55.3, 55.4 (C-5, OMe×2), 65.6 (C-27), 80.6 (C-3), 114.2, 114.3 (2C) (*p*-coumaroyl C-3), 116.0, 116.3 (*p*-coumaroyl C-β), 126.8 (C-12), 127.2, 127.3 (*p*-coumaroyl C-1, 129.6, 129.7 (2C) (*p*-coumaroyl C-2), 137.5 (C-13), 143.9, 144.3 (*p*-coumaroyl C-α), 161.2, 161.4 (*p*-coumaroyl C-4), 166.9, 167.0 (COO), 178.2 (C-28).

Hydrolysis of **1**.—A solution of **1** (102 mg) in 5% KOH/MeOH (7 ml) was refluxed overnight. The reaction mixture was diluted with H_2O, acidified with 1 N HCL, and extracted with EtOAc. The EtOAc layer was dried over Na_2SO_4 and concentrated to give a residue, which was subjected to Sephadex LH-20 cc. Elution with EtOH yielded a hydrolysate **4** (47 mg) as colorless needles (from MeOH): mp 240–242°; $[\alpha]^{20}D$ +53.3° (c=0.36, pyridine); negative fabms m/z $[M-H]^-$ 471; hrfabms m/z $[M+Na]^-$ 495.3453 ($C_{30}H_{48}O_4Na$, calcd 495.3450); ^1H nmr see Table 1; ^{13}C nmr see Table 2. Further elution with EtOH gave *p*-coumaric acid (30 mg).

Methylation of **4**.—A solution of **4** (20 mg) in $CHCl_3$/MeOH (2 ml) was treated with ethereal CH_2N_2 for 15 min. The reaction mixture was concentrated under reduced pressure to give a syrup, which was chromatographed on Si gel. Elution with hexane-EtOAc (3:1) afforded a methylate **5** (15 mg) as colorless prisms (from MeOH), which was found to be identical with methyl 3β,27-dihydroxyolean-12-en-28-oate by comparison of physical and spectral data with those described in the literature (2).

Asprellic acid B [**2**].—Colorless needles (from MeOH): mp 218–220°; $[\alpha]^{20}D$ +48.2° (c=0.28, pyridine); negative fabms m/z $[M-H]^-$ 763; hrfabms m/z $[M+Na]^+$ 787.4191 ($C_{48}H_{60}O_8Na$, calcd 787.4186); ^1H nmr see Table 1; ^{13}C nmr see Table 2.

Asprellic acid C [**3**].—A white amorphous powder; $[\alpha]^{20}D$ +90.0° (c=0.3, pyridine); negative fabms m/z $[M-H]^-$ 763; hrfabms m/z $[M+Na]^+$ 787.4192 ($C_{48}H_{60}O_8Na$, calcd 787.4186); ^1H nmr see Table 1; ^{13}C nmr see Table 2.

Hydrolysis of **2** *and* **3**.—A solution of **2** and **3** (10 mg each) in 5% KOH/MeOH was refluxed overnight. Workup as for **1** yielded **4** and *p*-coumaric acid.

BIOLOGICAL ASSAY.—The in vitro cytotoxicity assay was carried out according to a National Cancer Institute protocol (3) as previously described (4). The assay was conducted using a panel of human tumor cell lines. These cell lines are lung carcinoma (A-549), ileocecal adenocarcinoma (HCT-8), epidermoid carcinoma of the nasopharnyx (KB), melanoma (RPMI-7951), and leukemia (P-388). All cell lines were obtained from the American Type Culture Collection, Rockville, MD. In general, assay methods were the same as those described by Monks *et al.* (5). However, a tetrazolium salt method was used to measure cell survival and proliferation (6).

ACKNOWLEDGMENTS

The authors thank Drs. K. Mihashi and T. Fujioka, Faculty of Pharmaceutical Sciences, Fukuoka University, for mass spectral data. This investigation was supported by grant CA-17625 from the National Cancer Institute awarded to K.H. Lee.

LITERATURE CITED

1. X.M. Zhou, H.X. Chen, Y.C. Chen, and K.H. Lee, *J. Med. Chem.*, submitted.

2. J. Borges, J.C. Rodriguez, P. Vazquez, and P. Joseph, *J. Chem. Res. Synop.*, 130 (1983).
3. R.I. Geran, N.H. Greenberg, N.M. McDonald, A.M. Schumacher, and B.J. Abbott, *Cancer Chemother. Rep., Part 3*, **3**, 1 (1972).
4. K.H. Lee, Y.M. Lin, T.S. Wu, D.C. Zhang, T. Yamagishi, T. Hayashi, I.H. Hall, J.J. Chang, R.Y. Wu, and T.H. Yang, *Planta Med.*, **54**, 308 (1988).
5. A. Monks, D. Scudiero, P. Skehan, R. Shoemaker, K. Paull, D. Vistica, C. Hose, J. Langley, P. Cronise, A. Vaigro-Wolff, M. Gray-Goodrich, H. Campbell, J. Mayo, and M. Boyd, *J. Natl. Cancer Inst.*, **83**, 757 (1991).
6. T. Mosmann, *J. Immunol. Methods*, **65**, 55 (1983).

Received 10 May 1993