Phytoecdysteroids from *Ajuga macrosperma* var. *breviflora* Roots

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Three new phytoecdysteroids, ajugacetalsterones C (**1**) and D (**3**) and breviflorasterone (**2**), were isolated from the roots of *Ajuga macrosperma* var. *breviflora* along with five known compounds, namely, 20-hydroxyecdysone, cyasterone, makisterone A, 20-hydroxyecdysone 3-acetate, and 20-hydroxyecdysone 2-acetate. The structures of **1**−**3** were elucidated on the basis of extensive 1D and 2D NMR spectroscopic studies. The new compounds possess acetal oxygen bridges between C-26 and C-20/C-22, or C-26/C-23, or a lactone bridge between C-26 and C-23.

More than 100 species and 50 varieties and subspecies of *Ajuga* (Labiatae) are unevenly distributed over the world, being especially abundant in mainland China, Korea, and Japan, and are also widespread in Europe.[1−4] The genus *Ajuga* has attracted attention since *A. remota*, grown in Kenya, has been reported as not being attacked by African armyworms.[5] Thereafter, the isolation of *neo*-clerodane diterpenes, as the allelochemicals responsible for antifeedant activity from this genus, has been reported and reviewed.[6,7]

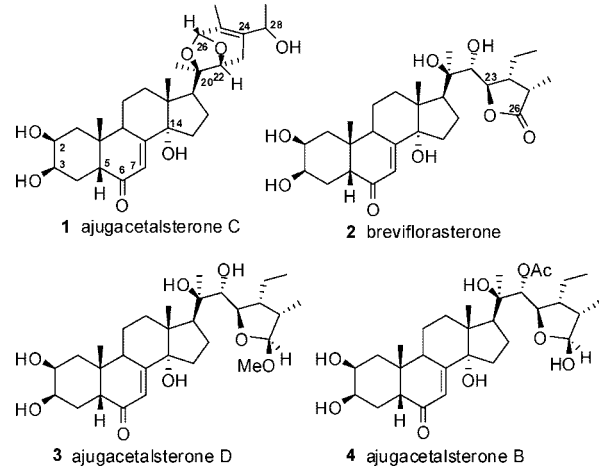
Ecdysteroids exhibit well-established physiological activities in insects (promote growth and control molting in insect metamorphosis) and also in mammals (beneficial effects on humans and animals)[8−11] and are also present in many representatives of the genus *Ajuga*.[2,6] Some of the successful applications of these plants in folk medicine might be explained by the presence of this type of metabolite.

Ajuga macrosperma Wall. ex Benth. is a perennial herb growing in tropical regions of India, Nepal, and the People's Republic of China.[12,13] Two varieties have been reported from mainland China, namely, *A. macrosperma* Wall. ex Benth. var. *macrosperma* and *A. macrosperma* var. *thomsonii*.[14] In China the herb *A. macrosperma* var. *macrosperma* is used in folk medicine to alleviate fever and remove phlegm,[14] and it is also reported to be used medicinally for nephritis.[3] The extract is active against *Pyricularia oryzae*[15] and also shows cell cycle inhibitory activity against the tsFT 210 cell line.[16] Our studies indicated that a root extract of *A. macrosperma* var. *breviflora* possessed antihelmintic activity against *Ascaridia galli*, a poultry roundworm.[17]

Previous phytochemical research on *A. macrosperma* resulted in the isolation of *neo*-clerodane diterpenoids (ajugamacrins A−E, as well as ajugacumbin B),[18,19] and the triterpenes betulinic and 3-*epi*-betulinic acids were isolated from a petroleum ether extract of aerial parts.[20]

A. macrosperma Wall. ex Benth. var. *breviflora* Hook. f. is reported to grow in the Kumaun region of Uttaranchal State in India.[12] We report herein the structure elucidation of three new ecdysteroidal compounds, named ajugacetalsterones C (**1**) and D (**3**) and breviflorasterone (**2**), isolated from *A. macrosperma* var. *breviflora* based on extensive NMR spectroscopic studies (standard one- and two-dimensional experiments).

The crude methanolic extract from roots of *A. macrosperma* afforded an ecdysteroid-containing fraction on filtering through a C_{18} RP column. The known ecdysteroids 20-hydroxyecdysone (the major one), cyasterone, makisterone A, 20-hydroxyecdysone 2-acetate, and 20-hydroxyecdysone 3-acetate were present along with three unknowns (Figure S1, Supporting Information). The residue was then filtered through a silica gel column, and pooled fractions, based on analytical TLC results, yielded compounds **1**−**3** on semipreparative HPLC conditions (Figures S2, S3, Supporting Information).



As shown in Table 1, the ^{1}H and ^{13}C NMR spectra of ajugacetalsterone C (**1**) showed characteristic ecdysteroid signals for protons/carbons 2, 3, 5, 7, 9, 19, and 21. The ^{13}C NMR spectrum displayed, in addition to the customary C-6 to C-8 sp^2 carbons, one set of two more in a C_{29} carbon frame, pointing to the presence of one extra double bond. Its location in the side chain (C-24, C-25) was supported by the singlet at δ 1.72 (H_3-27 signal) and some shifted signals [namely, slightly higher δ 4.56 for H-22 (doublet, J = 4.5 Hz) and slightly lower δ 1.02 for H_3-18 (singlet)]. Furthermore, supporting evidence for a Δ^{24} bond was derived from the HMBC spectrum (Table S1, Supporting Information) from δ 1.72 (H_3-27) to the δ 101.2 acetalic carbon (C-26) and with both sp^2 extra carbons. A δ 1.36 doublet (J = 6.6 Hz; H_3-29) pointed out the presence of a hydroxyethyl group as a C-24 substituent (further supported by the correlation δ 1.36/64.1 found in the HMBC spectrum). Moreover, the δ 5.46 acetalic proton signal displayed correlations with ^{13}C δ 85.3 (C-20) and 80.0 (C-22) signals, pointing out straightforwardly the presence of oxygen bridges C-26−O−C-20 and C-26−O−C-22. Thus, the structure was unambiguously established as **1**, showing the unprecedented dioxabicyclo[3.2.1]octene acetal function in the side chain.

The presence of an unsubstituted ethyl group as a C-28,C-29 fragment was concluded from the methyl (apparent) triplet at δ

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Table 1. NMR Spectroscopic Data of Compounds **1**−**3** (500 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR, in pyridine-d_5)[a]

	1		**2**		**3**	
position	δ_C	δ_H ($J/W_{1/2}$)	δ_C	δ_H ($J/W_{1/2}$)	δ_C	δ_H ($J/W_{1/2}$)
1a	37.5	1.91 t[b] (12.6)[c]	37.9	1.93 dd (13.4, 12.0)	37.9	1.82 dd (12.4, 12.2)
1e		2.10		2.14[c]		2.03[c]
2a	67.9	4.17 ($W_{1/2}$ = 21.2; d 10.3)	68.1	4.20 br d[d]	68.0	4.08 dt (11.3, 3.8)
3e	67.8	4.25 ($W_{1/2}$ = 9.4)	68.0	4.27 ($W_{1/2}$ = 8.4)	68.0	4.15 ($W_{1/2}$ = 8)
4a	32.2	1.83	32.5	1.83 br t[b] (13.4)	32.5	1.75[d]
4e		2.05 dt[b] (13.7, 3.7)		2.07[b] dt (13.7, 3.7)		2.01[c]
5a	51.1	3.01 dd (12.9, 3.1)	51.4	3.02 dd (13.5, 3.5)	51.4	2.93 dd (13.1, 3.7)
6	203.6		203.5			
7	121.4	6.25 d (1.8)	121.6	6.24 d (2.2)	121.4	6.16 d (2.2)
8	165.9		166.0			
9	34.1	3.57 ($W_{1/2}$ = 26.0)	34.4	3.60 m	34.4	3.50[c]
10	38.4		38.6		38.6	
11a	22.0	1.63	21.4[g]	1.72 q[b]d (12.0, 4.4)	21.1	1.64
11b		1.77		1.87[e]		
12e	31.3	1.90[e]	31.9	2.00 br d (12.4)	31.8	1.97
12a		2.50 t[b]d (13.0, 4.3)		2.60 t[b]d (12.4, 4.6)		2.56 t[b]d (13.9, 5.1)
13	47.4		48.1		47.7	
14	83.7		84.0		84.2	
15a	31.1	1.81	31.6	1.89[e]	31.8	1.77[d]
15b		1.86		2.15[c]		2.05[c]
16a	22.0	2.13	21.0[g]	2.15[e]	21.7	2.23[f]
16b		2.22 m		2.44 m		2.38 t[b]d (10.5, 8.8)
17	54.6	2.87 t[b] (8.8)	50.7	3.06 t[b] 9.0	50.1	3.48 dd (9.3, 8.8)[e]
18	16.4	1.02 s	18.0	1.19 s	18.2	1.13 s
19	24.1	1.03 s	24.4	1.07 s	24.4	0.97 s
20	85.3		75.8		76.4	
21	23.2	1.66 s	22.5	1.65 s	22.8	1.59 s
22	80.0	4.56 d (4.5)	77.8	4.19 br s[d]	79.4	3.77 d (8.0)
23a	26.0	2.67 d_{AB} (17.6)	82.9	4.62 br d (2.4)	83.4	3.96 t[b] (8.0)
23b		2.75 d_{AB} (17.6)				
24	132.4		40.9	2.81 t[b]d (9.0, 4.5)	47.7	2.50 m
25	129.8		38.1	3.45 quint[b] (7.7)	41.0	2.24 quint[b] (6.8)[f]
26	101.2	5.46 s	180.4		111.6	4.57 s
27	14.7	1.72 s	10.6	1.14 d (7.6)	11.6	0.76 d (7.1)
28a	64.1	4.95	22.0	1.22 m	21.3	1.33
28b				1.48 m		2.07[e]
29	21.0	1.36 d (6.6)	12.0	0.98 t[b] (7.3)	13.0	0.78 t[b] (7.3)
OMe					55.4	3.40 s
OH		6.35 br		6.40 br, 7.35 br		

[a] Chemical shift values in δ (ppm); coupling constants/bandwidth at half-height $J/W_{1/2}$ (Hz) in parentheses; solvent central bands as reference (^{1}H 7.55 ppm and ^{13}C 135.5 ppm). [b] Apparent signals (t[b] = dd and quint[b] = dq with $J_1 \approx J_2$; q[b] = ddd with $J_1 \approx J_2 \approx J_3$). [c–f] Overlapping signals: same superscript in any column. [g] Interchangeable assignments.

0.98 (J = 7.3 Hz) in the ^{1}H NMR spectrum of breviflorasterone (**2**) (Table 1), along with three characteristic methyl singlets (δ 1.07, H_3-19; δ 1.19 H_3-18; and δ 1.65 H_3-21) and one doublet (δ 1.14, J = 7.6 Hz; H_3-27). The COSY spectra showed the following coupling sequence: δ 1.14 (doublet J = 7.6 Hz) ↔ 3.45 [multiplet (apparent quintet, J = 7.7 Hz)] ↔ 2.81 [(apparent) triplet of doublets, J = 9.0 and 4.5 Hz] ↔ 4.62 [doublet, J = 2.4 Hz] ↔ 4.19 [(apparent) singlet] for the spin system H_3-27 ↔ H-25 ↔ H-24 ↔ H-23 ↔ H-22. Thus, the highly deshielded δ 4.62 doublet led to the conclusion of an oxygen substitution at C-23. Furthermore, a ^{13}C NMR signal at δ 180.4 was consistent with the presence of a carbonyl lactone. Formation of a 26,23-lactone was substantiated by the HMBC correlations of protons at δ 4.62, 3.45, and 1.14 with C-26. Thus, the structure assigned to **2** displays an unprecedented 26,23-lactone group in the side chain.[21,22]

On the plausible assumption of a common biosynthesis, the relative configurations of C-24 and C-25 were considered the same as for cyasterone[23] (in **2** C-24*R* and C-25*S*), but a NOESY experiment gave inconclusive results. Regarding the C-23 assignment, dihedral angles H-22−H-23, H-23−H-24, and H-24−H-25 were estimated on simplified models (MM2 energy minimization by Chem3D program) for both C-23*R* and C-23*S* configurations (Figure S4, Supporting Information). Results for the C-23*R* configuration better support a very small H-22−H-23 coupling constant, as found, whereas the predicted coupling constants for H-23−H-24 are quite similar for both.

As shown in Table 1, five methyl signals and one methoxyl signal [one δ 0.76 doublet (J = 7.1 Hz), one δ 0.78 (apparent) triplet (J = 7.3 Hz), and singlets at δ 0.97 (H_3-19), 1.13 (H_3-18), 1.59 (H_3-21), and 3.40 ppm (OMe)] were displayed in the ^{1}H NMR spectrum of ajugacetalsterone D (**3**). Another singlet at δ 4.57 ppm pointed out the likely presence of an acetal group [H−C(C,O,O)] in the side-chain moiety. In agreement with the assignment of the last two singlets, the ^{13}C NMR spectrum displayed signals at δ 55.4 and 111.6. The presence of four signals close to 80 ppm, instead of the usual three (C-14, C-20, and C-22), required one extra oxygen-bonded carbon. The ^{1}H−^{13}C multiple-bond correlation NMR spectrum (Table S1, Supporting Information) showed a cross-signal between an oxygen-bonded carbon (δ 83.4) and the acetal proton signal and vice versa, pointing out the presence of an oxygen bridge across C-26 and C-23. Further correlations from both methyl multiplets secured the substitution pattern and assignments of the side chain as shown in Figure S1. Dihedral angles H-22−H-23, H-23−H-24, H-24−H-25, and H-25−H-26 for both C-23*R* and C-23*S* configurations were derived from the conveniently modified models (MM2 energy minimization by Chem3D program). The predicted values for C-23*R* are in good agreement with the experimental findings, but point to a conformational change around the C-22−C-23 bond. We assume the same configuration may be present in the stereogenic side-chain carbons for **2**, **3**, and the previously reported ajugacetalsterone B (**4**).[24,25]

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer model 341 polarimeter. NMR spectra were obtained on Varian Inova 500/Mercury 400 spectrometers (500 MHz for ^{1}H and 100 MHz for ^{13}C, respectively) in 350 μL of pyridine-d_5, using Shigemi microtubes (BMS-005B matched with D_2O), under standard 1D and 2D conditions and pulse sequences. MS were measured on a Waters ACQUITY UPLC with a Q-TOF Premier mass spectrometer detector. The HPLC separation work was performed on a Waters Alliance 2695 apparatus coupled with a 996 UV diode array detector, and fractions were collected on a Waters fraction collector III. Solvents used for extraction and chromatographic procedures were HPLC grade, and water used in mobile phase mixtures was Milli Q (Millipore R, Billerica, MA). Chromagel 60 Å 35−70 μm silica gel was used for column chromatography (SDS, Val de Reuil, France), and 60 F_{254} aluminum sheets (Merck, Darmstadt, Germany) were used for TLC analysis.

Plant Material. The plant *A. macrosperma* var. *breviflora* was collected from Ramnagar region (29°26′19.27″ N and 79°06′07.22″ E) of Uttarakhand State, India, during March 2004. The plant was identified by Prof. Y. P. S. Pangty, Taxonomist, Kumaun University, Nainital, and a voucher specimen (herbarium number Chem 1504) was deposited at the Chemistry Department, G. B. Pant University of Agric. & Tech., Pantnagar, India. Plants were dried in the shade.

Extraction and Isolation. Dried and powdered roots (400 g) were extracted with MeOH (2.0 L, ×3) at room temperature. The extracts were combined and evaporated under vacuum to give 43 g of a red-brown extract. The crude extract (10 g) was sonicated with a H_2O−MeOH mixture (85:15 v/v; 50 mL, ×2). The resulting suspension was centrifuged (1500*g*, 15 min), and the supernatant was filtered through a C_{18} RP column [Isolute SPE C18 10 g cartridge (IST, Tucson, AZ)]. The column was first activated/equilibrated (30 mL of MeOH followed by 30 mL of H_2O−MeOH, 85:15) and eluted with H_2O−MeOH (85:15, 100 mL and 15:85, 100 mL) afterward. This last fraction was collected, evaporated to dryness (1.47 g of residue), resuspended in 7.5 mL of $CHCl_3$−MeOH (9:1 v/v), mixed with 3 g of silica gel, and placed on top of a 12 g silica gel column after drying. The column was eluted with $CHCl_3$−MeOH mixtures (98:2, 60 mL; 95:5, 100 mL; 9:1, 45 mL; 85:15, 40 mL; 4:1, 30 mL; 0:1, 40 mL). Fractions (collected every 5 mL) were analyzed by TLC ($CHCl_3$−MeOH−H_2O, 44:9:1) and combined from 33 to 37 and from 42 to 44 (ecdysteroid-containing fractions). Compounds **3** (system 1) and **1** and **2** (system 2) were then isolated on semipreparative HPLC {Kromasil 100 C18, 5 μm 25 × 1 cm column [Teknokroma, San Cugat del Vallés, Spain], and a C_{18} guard column used to protect its integrity, H_2O−acetonitrile mixtures used as mobile phases [70:30 (7 min), 70:30 to 65:35 in 5 min, 65:35 (25 min), 65:35 to 70:30 in 3 min (system 1) or 65:35 (10 min), 65:35 to 50:50 in 15 min, 50:50 to 65:35 in 5 min (system 2)], at 25 °C, a flow rate of 2.0 mL/min, and fractions collected every 30 s}. MS were obtained by positive ESI (being the resulting mass M + 1) from pure compound solutions [5 μL of 0.1 mg/mL was injected on an Acquity UPLC BEH C18 1.7 μm 2.1 × 100 mm column, H_2O−acetonitrile containing 0.1% v/v HCO_2H (**1**, **2**: 80:20; **3**: 65:35) as mobile phases, 30 °C, 0.3 mL/min flow] and referred externally for accurate mass results.

Ajugacetalsterone C (1): white, amorphous solid (0.4 mg); $[\alpha]^{22}_D$ +253.9 (*c* 0.04, MeOH); ^{1}H NMR and ^{13}C NMR data, Table 1; HRESIMS *m/z* 503.3004 [M + 1] (calcd for $C_{29}H_{43}O_7$, 503.3009).

Breviflorasterone (2): white, amorphous solid (0.8 mg); $[\alpha]^{22}_D$ −159.3 (*c* 0.23, MeOH); ^{1}H NMR and ^{13}C NMR data, Table 1; HRESIMS *m/z* 521.3140 [M + 1] (calcd for $C_{29}H_{45}O_8$, 521.3114).

Ajugacetalsterone D (3): white, amorphous solid (1.3 mg); $[\alpha]^{22}_D$ +71.6 (*c* 0.10, MeOH); ^{1}H NMR and ^{13}C NMR data, Table 1; HRESIMS *m/z* 537.3428 [M + 1] (calcd for $C_{30}H_{49}O_8$: 537.3427).

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Supporting Information Available: Table S1 shows NMR data including HMBC for **1**−**3** and Table S2 the ecdysteroid identification/quantification (based on relative retention times, RR_t, in different chromatographic systems). Figures S1−S3 show analytical and semi-preparative HPLC profiles of *A. macrosperma* enriched ecdysteroid fraction and Figure S4 a model energy minimization for the 23*R* and 23*S* configuration This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Ball, P. W. In *Flora Europaea III. Diapensiaceae to Myoporaceae*; Tutin, T. G., Heywood, V. H., Burges, N. A., Moore, D. M., Valentine, D. H., Walters, S. M., Webb, D. A., Eds.; Cambridge University Press: Cambridge, 1972; pp 128−129.
(2) Darvas, B. *Növényvédelem* **1991**, *27*, 481–498.
(3) *Flora of China*; 1994; p 69, www.efloras.org.
(4) *The International Plant Names Index*; 2004, www.ipni.org.
(5) Kubo, I.; Lee, Y. W.; Balogh-Nair, V.; Nakanishi, K.; Chapya, A. *J. Chem. Soc., Chem. Commun.* **1976**, 949–950.
(6) Camps, F.; Coll, J. *Phytochemistry* **1993**, *32*, 1361–1370.
(7) Coll, J.; Tandrón, Y. *Phytochem. Rev.* **2008**, *7*, 25–49.
(8) Koolman, J. *Ecdysone. From Chemistry to Mode of Action*; Thieme Medical Publishers, Inc.: New York, 1989.
(9) Lafont, R.; Dinan, L. *J. Insect Sci.* **2003**, *3* (7), available online at insectscience.org/3.7.
(10) Bathori, M.; Pongracz, Z. *Curr. Med. Chem.* **2005**, *12*, 153–172.
(11) Lafont, R.; Harmatha, J.; Marion-Poll, F.; Dinan, L.; Wilson, I. D. *The Ecdysone Handbook*; 2002, http://www.ecdybase.org.
(12) Strachey, R. *Flora of Kumaun, Garhwal and Adjoining Areas of Tibet*; Bishan Singh and Sons: Dehradun, India, 1974.
(13) Babu, C. R. *Herbaceous Flora of Dehradun; Publication and Information Directorate*; CSIR: New Delhi, 1977; p 405.
(14) Yunnan Institute of Botany, Ed. *Flora Yunnanica*; Tomus Wall Science Press: Peking, 1977.
(15) Hu, K.; Dong, A.; Liu, H.; Feng, H.; Sun, Q.; Yao, X. *Pharm. Biol.* **1999**, *37*, 225–230.
(16) Qingchun, Z.; Chengbin, C.; Bing, C.; Feng, Q.; Tao, G.; Xinsheng, Y. *Pharm. Biol.* **2005**, *43*, 135–139.
(17) Agarwal, J.; Prakash, O.; Pant, A. K.; Hore, S. K. Unpublished data.
(18) Shen, X.; Isogai, A.; Furihata, K.; Sun, H.; Suzuki, A. *Phytochemistry* **1993**, *33*, 887–889.
(19) Shen, X.; Isogai, A.; Furihata, K.; Sun, H.; Suzuki, A. *Phytochemistry* **1993**, *34*, 1091–1094.
(20) Dhinda, B.; Banerjee, J.; Guha, S. *J. Indian Chem. Soc.* **1997**, *74*, 424.
(21) Unprecedented among ecdysteroid compounds. However the isolation of withanolides displaying a closely related side chain, such as 2,3-dihydro-3β-methoxyixocarpalactone A,[22] was brought to our attention by a referee.
(22) Gu, J.-Q.; Li, W.; Kang, Y.-H.; Su, B.-N.; Fong, H. H. S.; van Breemen, R. B.; Pezzuto, J. M.; Douglas Kinghorn, A. *Chem. Pharm. Bull.* **2003**, *51*, 530–539.
(23) Okuzumi, K.; Hara, N.; Uekusa, H.; Fujimoto, Y. *Org. Biomol. Chem.* **2005**, *3*, 1227–1232.
(24) Coll, J.; Tandrón, Y. A.; Zeng, X. *Steroids* **2007**, *72*, 270–277.
(25) Ajugacetalsterone B should be better represented as **4** rather than as reported previously.[24]

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