# Ajugins C and D, New Withanolides from *Ajuga parviflora*

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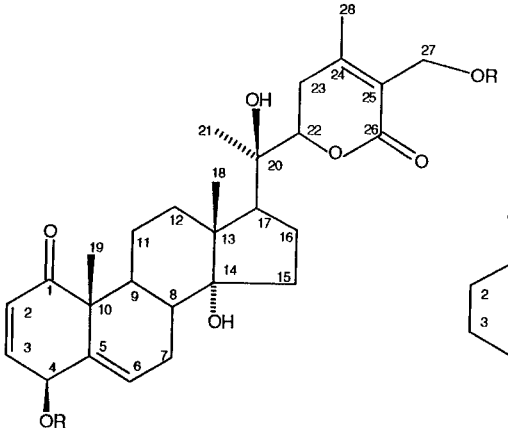
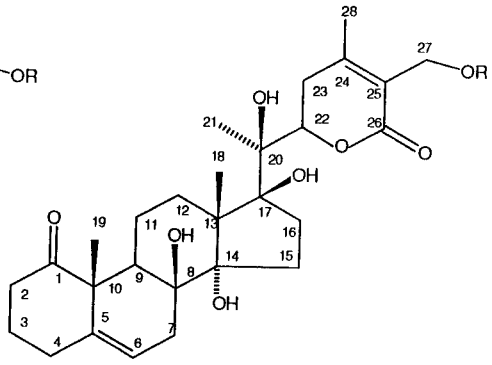
The new withanolides ajugin C (=(20*R*,22*R*)-4$\beta$,14$\alpha$,20,27-tetrahydroxy-1-oxoergosta-2,5,24-trieno-26,22-lactone; **1**) and ajugin D (=(20*R*,22*R*)-8$\beta$,14$\alpha$,17$\beta$,20,27-pentahydroxy-1-oxoergosta-5,24-dieno-26,22-lactone; **2**) were isolated from the whole plant of *Ajuga parviflora*. Their structures were determined by spectroscopy, including 2D-NMR.

**1. Introduction.** – Many plants of the genus *Ajuga* (Labiatae) are used in local medicine as antifebrile, anthelmintic, vulnerary, and hypoglycaemic agents. A number of neoclerodane diterpenoids and ecdysteroids obtained from them have shown antitumoral, antimicrobial, antifungal, insect antifeedant, and hypoglycaemic activities [1][2]. *Ajuga parviflora* is found in northern areas of Pakistan and is locally used against fever, stomach disorder, and throat diseases. Recently [3], we reported the isolation of ajugins A (=(20*R*,22*R*)-14$\alpha$,20,28-trihydroxy-1-oxowitha-3,5,24-trienolide =(20*R*,22*R*)-14$\alpha$,20,28-trihydroxy-1-oxoergosta-3,5,24-trieno-26,22-lactone) and B (=(20*R*,22*R*)-14$\alpha$,20,27-trihydroxy-1-oxowitha-5,24-dienolide=(20*R*,22*R*)-14$\alpha$,20,27-trihydroxy-1-oxoergosta-5,24-dieno-26,22-lactone), the first report of natural occurrence of withanolides in the Labiatae family. This paper deals with the isolation and structure elucidation of two further withanolides, ajugin C (**1**) and D (**2**).



**1** R = H; **ajugin C**
**1a** R = Ac



**2** R = H; **ajugin D**
**2a** R = Ac

**2. Results and Discussion.** – A crude plant extract of *Ajuga parviflora* was obtained with 90% MeOH/$H_2O$. The extract was defatted by hexane extraction and finally partitioned between AcOEt and $H_2O$. The AcOEt fraction was prefractionated by column and flash chromatography over silica gel with different mobile phases. Ajugin C (**1**) and D (**2**) were finally obtained by low-pressure LC (silica gel) and prep. TLC (silica gel), and their structures were established by UV, IR, NMR spectroscopy, and mass spectrometry, and by the spectroscopic data of their acetylation products **1a** and **2a**, respectively.

The HR-FAB-MS (positive-ion mode) of **1** provided $[M+H]^+$ at *m/z* 487.2707, indicating the molecular formula $C_{28}H_{38}O_7$. The peak at *m/z* 141.0653 ($C_7H_9O_3$) resulting from the cleavage of the C(20)–C(22) bond suggested the presence of a six-membered hydroxy-lactone at C(20) [4]. The UV spectrum showed an absorption at $\lambda_{max}$ 219 nm. The sizeable blue shift compared to the characteristic absorption (225 nm) of the usual dimethyl-substituted $\alpha,\beta$-unsaturated $\delta$-lactone of the withanolides indicated its presence with an $\alpha$-hydroxymethyl substitution [5]. The IR spectrum revealed the presence of several OH groups (3560 and 3405 $cm^{-1}$), and included bands at 1692 and 1708 $cm^{-1}$ typical for a six-membered cyclic ketone and an $\alpha,\beta$-unsaturated $\delta$-lactone, respectively. The $^1$H-NMR spectrum of **1** closely resembled that of 5,6-deoxywithaferin A revealing the same substitution pattern in rings A and B [6]. The spectrum included signals for three tertiary Me groups ($\delta$ 1.54, 1.32, and 1.04) and one vinylic Me group ($\delta$ 1.98). The absence of a signal corresponding to Me(27) and the presence of a 2-H *ABd* pattern at $\delta$ 4.73 and 4.68, which was shifted downfield to $\delta$ 5.23 and 5.18 in diacetate **1a**, suggested a substitution with a OH group at C(27) [6]. The olefinic signals at $\delta$ 6.69 (*dd*, $J=10.0$, 4.5 Hz, 1 H), 5.73 (*d*, $J=9.6$ Hz, 1 H), and 5.50 (br. *d*, $J=5.5$ Hz, 1 H) were attributed to H–C(3), H–C(2), and H–C(6), respectively, and the two oxymethine protons at $\delta$ 4.62 (br. *d*, $J=4.3$ Hz, 1 H) and 4.44 (*dd*, $J=$ 12.5, 3.6 Hz, 1 H) to H–C(4) and H–C(22), respectively. The latter exhibited cross-peaks in the COSY experiment with the $CH_2$(23) signals at $\delta$ 2.42 and 2.08. The multiplicity of H–C(22) (*dd*) and the location of Me(21) as a *s* were indicative of a 20-OH group. It has been reported that when C(22) has (*S*)-configuration, H–C(22) resonates as a broad *s* with $w_{1/2} \approx 5$ Hz, while in the (22*R*)-isomer, it appears as a *dd* with two coupling constants characteristic for axial-axial and axial-equatorial interactions with $CH_2$(23) [7]. On this basis, (*R*)-configuration was assigned to C(22). Since all the double-bond equivalents and six out of seven O-atoms have already been accounted for, the remaining O-atom must be present as tertiary OH group, which was placed at C(14) because of the downfield shift of the C(14) signal [8] to $\delta$ 84.7. The position of OH at C(14) was further confirmed by a HMBC experiment which showed a $^2J$ correlation of C$H_3$(18) ($\delta$ 1.04) to C(13) ($\delta$ 49.8) and a $^3J$ correlation to C(12) ($\delta$ 25.0), C(14) ($\delta$ 84.7), and C(17) ($\delta$ 49.1). It has been observed that a 14$\beta$-OH group does not cause shielding of C(12) [9], while 14$\alpha$-OH shields C(7), C(9), and C(12) through the $\gamma$-effect and deshields C(8) through the $\beta$-effect [10]. On this basis, $\alpha$-orientation was assigned to the 14-OH group. The $^{13}$C-NMR spectrum of **1** showed 28 C-signals, and their shift values corroborated the above substitution pattern. Assignments of all the functional groups were confirmed by HMQC and HMBC experiments and comparison with related withanolides [4][6].

Ajugin D (**2**) displayed a $[M+H]^+$ peak at *m/z* 505.3803 in the HR-FAB-MS (positive-ion mode), corresponding to the molecular formula $C_{28}H_{40}O_8$. The UV absorption at 220 nm revealed the presence of an $\alpha,\beta$-unsaturated $\delta$-lactone [11]. The IR spectrum showed absorptions at 3470, 1715, and 1696 $cm^{-1}$ for OH, ketone C=O and $\alpha,\beta$-unsaturated $\delta$-lactone moieties, respectively [12]. The ring A of **2** differed from that of **1** in lacking C=C and OH functionalities and gave rise to only one olefinic signal at $\delta$ 5.30 assignable to H–C(6). However, the signal of C=O at $\delta$ 217.8 in the $^{13}$C-NMR showed a similar chemical shift to that reported for withametelin-$H_2$ [13] and could be attributed to C(1). Comparison of $^{13}$C-NMR data with that of withametelin-$H_2$ having a similar ring A established the substitution pattern of both rings A and B [13]. The $^1$H-NMR, $^{13}$C-NMR, and MS data of **2** revealed the same $\delta$-lactone moiety as in **1**. Out of eight O-atoms, five have already been accounted for, the remaining three O-atoms appeared to be present as tertiary OH groups. This was supported by the presence of three signals for quaternary C-atoms at $\delta$ 79.1, 83.0, and 87.4 in the $^{13}$C-NMR spectrum. Their positions were determined by HMBC experiments. There was a $^3J$ correlation from $\delta$ 5.30 (H–C(6)) to $\delta$ 79.1 (C), confirming the position of one of the OH groups at C(8); OH–C(8) was assigned $\beta$-orientation on biogenetic grounds and also by comparing $^{13}$C-NMR chemical shifts with those of similarly substituted withanolides [14]. An OH group was assigned to C(14) by observing a $^2J$ correlation of C$H_3$(18) ($\delta$ 1.07) to C(13) ($\delta$ 54.2) and a $^3J$ correlation to C(12) ($\delta$37.1), C(14) ($\delta$ 83.0), and C(17) ($\delta$ 87.4); OH–C(14) was

assigned $\alpha$-orientation due to the shielding of C(7), C(9), and C(12) through $\gamma$-effects and by comparison with related withanolides [15]. The remaining OH group was placed at C(17). In the HMBC, C(17) showed a $^3J$ correlation with $\delta$ 1.07 (Me(18)) and $\delta$ 1.43 (Me(21)). The $\beta$-configuration of OH–C(17) could be deduced from the characteristic pyridine-induced downfield shift for Me(18), as has been observed with the 17$\beta$-hydroxywithanolides [16][17].

## Experimental Part

*General*. Column chromatography (CC): silica gel, 70–230 mesh. Flash chromatography (FC): silica gel 220–440 mesh. TLC: pre-coated silica-gel *G-25-UV$_{254}$* plates; detection at 254 and 366 nm, and by *Dragendorff*'s reagent. Optical rotations: *Jasco-DIP-360* polarimeter. UV and IR Spectra: *Hitachi-UV-3200* and *Jasco-302-A* spectrophotometers, resp. $^1$H- and $^{13}$C-NMR, COSY, HMQC, and HMBC Spectra: *Bruker* spectrometers operating at 500 and 400 MHz; chemical shifts $\delta$ in ppm rel. to $SiMe_4$ as internal standard and coupling constants in Hz. EI-, FAB-, and HR-EI-MS: *JMS-HX-110* with a data system and *JMS-DA-500* mass spectrometers; *m/z* (rel. int).

*Plant Material*. The whole plant of *Ajuga parviflora* BENTH (Labiatae), collected from Swat (Pakistan) in July, 1997, was identified by Dr. *Jahandar Shah*, plant taxonomist, Department of Botany, University of Peshawar. A voucher specimen was deposited in the herbarium (PUH-14918) of Peshawar University.

*Extraction and Isolation*. The air-dried ground plant (20 kg) was exhaustively extracted with 90% MeOH at r.t. The extract was evaporated and the residue (1.2 kg) defatted by extraction with hexane. The defatted extract was partitioned between AcOEt and $H_2O$. The AcOEt extract was submitted to CC (hexane/AcOEt and AcOEt/MeOH gradients). The fractions obtained with AcOEt/MeOH 9:1 were subjected to FC (AcOEt and AcOEt/MeOH gradient). Fractions obtained with AcOEt/MeOH 8.5:1.5 were combined and further subjected to low-pressure liquid chromatography (*Lobar LiChroprep Si 60*, *Merck*) column, AcOEt/MeOH 9.4:0.8). Final purification of the resulting fractions by prep. TLC ($CHCl_3$/$C_6H_6$/MeOH/$H_2O$ 3.8:3.8:4.4:0.5) afforded pure compounds **1** and **2**.

*Ajugin C* (=*(20*R,*22*R*)-4β,14α,20,27-Tetrahydroxy-1-oxowitha-2,5,24-trienolide* = *(20*R,*22*R*)-4β,14α,20,27-Tetrahydroxy-1-oxoergosta-2,5,24-trieno-26,22-lactone*; **1**): Amorphous solid (28 mg). M.p. 203–204°. $R_f$ 0.45. $[\alpha]_D^{25} = +123$ ($c = 0.28$, MeOH). UV (MeOH): 219. IR (KBr): 3560, 3405, 1708, 1692. $^1$H-NMR ($CDCl_3$ + $CD_3OD$, 400 MHz): 6.69 (*dd*, $J = 10.0$, 4.5, H–C(3), 5.73 (*d*, $J = 9.6$, H–C(2)); 5.50 (br. *d*, $J = 5.5$, H–C(6)); 4.62 (*d*, $J = 4.3$, H–C(4)); 4.73, 4.68 (*ABd*, $J = 10.8$, $CH_2$(27)); 4.44 (*dd*, $J = 12.5$, 3.6, H–C(22)); 1.98 (*s*, Me(28)); 1.54 (*s*, Me(19)); 1.32 (*s*, Me(21)); 1.04 (*s*, Me(18)). $^{13}$C-NMR ($CDCl_3$ + $CD_3OD$, 125 MHz): 202.2 (C(1)); 166.7 (C(26)); 154.1 (C(24)); 143.1 (C(3)); 131.0 (C(2)); 129.8 (C(5)); 125.8 (C(6)); 125.0 (C(25)); 84.7 (C(14)); 81.7 (C(22)); 75.0 (C(20)); 68.1 (C(4)); 55.8 (C(27)); 50.7 (C(10)); 49.8 (C(13)); 35.9 (C(9)); 35.1 (C(8)); 32.1 (C(7)); 32.0 (C(23)); 31.7 (C(16)); 31.6 (C(15)); 25.0 (C(12)); 22.0 (C(11)); 22.6 (C(19)); 21.3 (C(21)); 20.4 (C(28)); 17.8 (C(18)). HR-FAB-MS (pos.): 487.2707 ($[M+H]^+$). EI-MS: 468 (7), 450 (5), 283 (17), 185 (24), 167 (8) (7), 141 (92), 124 (100).

*(20*R,*22*R*)-4β,27-Bis(acetyloxy)-14α,20-dihydroxy-1-oxoergosta-2,5,24-trieno-26,22-lactone* (**1a**). Compound **1** (16 mg) was acetylated with $Ac_2O$ (2 ml) in pyridine (2 ml) at r.t. for 24 h. Usual workup and prep. TLC afforded **1a** (12 mg). $[\alpha]_D^{25} = +112$ ($CHCl_3$, $c = 0.31$). UV (MeOH): 222. IR ($CHCl_3$): 3454, 1735, 1712, 1703. $^1$H-NMR ($CDCl_3$, 400 MHz): 6.72 (*dd*, $J = 10.2$, 4.2, H–C(3)); 6.02 (*d*, $J = 9.4$, H–C(2)); 5.86 (br. *d*, $J = 4.2$, H–C(4)); 5.58 (br. *d*, $J = 5.4$, H–C(6)); 5.23, 5.18 (*ABd*, $J = 10.4$, $CH_2$(27)); 4.45 (*dd*, $J = 12.8$, 3.4, H–C(22)); 2.06 (*s*, AcO); 2.04 (*s*, AcO); 1.89 (*s*, Me(28)); 1.34 (*s*, Me(21)); 1.57 (*s*, Me(19)); 1.03 (*s*, Me(18)). EI-MS: 570 (5, $M^+$), 510 (10), 450 (13), 183 (12), 167 (20), 124 (92).

*Ajugin D* (=*(20*R,*22*R*)-8β,14α,17β,20,27-Pentahydroxy-1-oxowitha-5,24-dienolide* = *(20*R,*22*R*)-8β,14α,17β,20,27-Pentahydroxy-1-oxoergosta-5,24-dieno-26,22-lactone*; **2**). White solid (20 mg). M.p. 273–275°. $R_f$ 0.33. $[\alpha]_D^{25} = +74$ ($CHCl_3$, $c = 0.39$). UV (MeOH): 220. IR (KBr): 3470, 1715, 1696. $^1$H-NMR ($CDCl_3$ + $CD_3OD$, 400 MHz): 5.30 (*d*, $J = 5.3$, H–C(6)); 4.61 (*dd*, $J = 13.0$, 3.5, H–C(22)); 4.42, 4.35 (*ABd*, $J = 12.6$, $CH_2$(27)); 2.01 (*s*, Me(28)); 1.42 (*s*, Me(21)); 1.22 (*s*, Me(19)); 1.07 (*s*, Me(18)). $^{13}$C-NMR ($CDCl_3$ + $CD_3OD$, 125 MHz): 217.8 (C(1)); 168.0 (C(26)); 157.3 (C(24)); 142.1 (C(5)); 126.0 (C(25)); 124.0 (C(6)); 87.4 (C(17)); 83.2 (C(22)); 83.0 (C(14)); 79.1 (C(8)); 78.7 (C(20)); 56.4 (C(27)); 54.2 (C(13)); 50.6 (C(10)); 39.9 (C(9)); 38.9 (C(2)); 37.3 (C(16)); 37.1 (C(12)); 32.9 (C(15)); 31.6 (C(4)); 32.0 (C(7)); 30.7 (C(23)); 31.5 (C(4)); 26.8 (C(3)); 21.7 (C(11)); 21.3 (C(21)); 20.4 (C(28)); 19.1 (C(19)); 16.2 (C(18)). HR-FAB-MS (pos.): 505.3803 ($[M+H]^+$). EI-MS: 486 (6), 468 (10), 301 (15), 185 (68), 167 (24), 141 (100), 125 (90).

*(20*R,*22*R*)-27-(Acetyloxy)-8β,14α,17β,20-tetrahydroxy-1-oxoergosta-5,24-dieno-26,22-lactone* (**2a**). Acetylation of **2** was carried out as described for **1a**. **2a**: $[\alpha]_D^{25} = 45$ ($CHCl_3$, $c = 0.34$). UV (MeOH) 226. IR ($CHCl_3$):

3405, 1734, 1710, 1692. $^1$H-NMR ($CDCl_3$, 400 MHz): 5.48 (*d*, *J* = 5.4, H–C(6)); 4.62 (*dd*, *J* = 12.8, 3.4, H–C(22)); 4.95, 4.90 (*ABd*, *J* = 12.5, $CH_2$(27)); 2.14 (*s*, COMe); 2.03 (*s*, H–C(28)); 1.44 (*s*, H–C(21)); 1.24 (*s*, H–C(19)); 1.27 (*s*, H–C(18)). EI-MS: 546 (5, $M^+$), 486 (8), 368 (10), 183 (20), 125 (94).

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