PERGAMON

Phytochemistry 52 (1999) 1357–1360

PHYTOCHEMISTRY

Ajugin E and F: Two withanolides from *Ajuga parviflora*

Hafiz Rub Nawaz, Abdul Malik*, Pir Mohammad Khan, Saeed Ahmed

International Centre for Chemical Sciences, HEJ Research Institute of Chemistry, University of Karachi, Karachi 75270, Pakistan

Received in revised form 2 June 1999; accepted 2 June 1999

Abstract

Two withanolides, Ajugin E and F, were isolated from the defatted methanolic extract of *Ajuga parviflora*. Their structures were established via spectroscopic analysis, including high resolution one- and two-dimensional NMR spectrometry as 14α,17β,20,27-tetrahydroxy-1-oxo-(20*R*, 22*R*)-witha-3,5,24-trienolide **1**, and 14α,17α,27-trihydroxy-1-oxo-(20*R*, 22*R*)-witha-5,24-dienolide **2**, respectively.

Keywords: *Ajuga parviflora*; Labiatae; Withanolides; Ajugin E and F

1. Introduction

Species belonging to genus *Ajuga* (Labiatae) have been used as folk medicinal plants as anthelmintic, antifungal, hypoglycaemic, antitumor and antimicrobial agents (Rodriguez-Hahn, Esquivel & Cardenas, 1994; Wessner, Champion, Girault, Kaouadji, Saidi & Lafont, 1992). During a search for new bioactive compounds from medicinal plants, we found that the defatted methanolic extract of *Ajuga parviflora*, an annual or short lived perennial herb growing in the hilly regions of northern Pakistan, showed strong brine shrimp bioactivity. This encouraged us to study the chemical constituents of this plant. The isolation of Ajugin A to D from *A. parviflora* has recently been reported from our laboratories (Khan, Ahmad, Nawaz & Malik, in press). In this paper, we now report the isolation and structural elucidation of two new withanolides, Ajugin E (**1**) and F (**2**).

2. Results and discussion

Ajugin E (**1**) showed absorptions indicative of hydroxyl groups, a six membered cyclic ketone and α,β-unsaturated δ-lactone in its IR spectrum. The UV spectrum was characteristic of withanolides showing the absorption at λ_{max} 223 nm attributable to α,β-unsaturated δ-lactone (Pavia, Lampman & Kriz, 1979). The high resolution FAB mass spectrum showed ion $[M + H]^+$ peak at *m/z* 487.2697 corresponding to the molecular formula $C_{28}H_{38}O_7$. The EI mass spectrum showed diagnostic peaks at *m/z* 468 $[M^+–H_2O]$, 450 $[M^+–2H_2O]$, 301 [M^+-side chain], and 283 [M^+-side chain–H_2O]. The ion peak at *m/z* 185 resulting from the cleavage of C-17/C-20 bond indicated the presence of a hydroxyl group at C-20 while another peak at *m/z* 141 was due to hydroxy substituted α,β-unsaturated δ-lactone which is formed by the cleavage of C-20/C-22 bond (Ramaiah, Lavie, Budhiraja, Sudhir & Garg, 1984). The ^{1}H-NMR spectrum of **1** closely resembled to that of isowithanolide F (Velde, Lavie, Budhiraja, Sudhir & Garg, 1983) and indicated the presence of a 3,5-diene-1-oxo system in rings A and B of the steroidal skeleton. It included signals for two mutually coupled olefinic protons at δ 5.76 and δ 5.90 assignable to C-3 and C-4 vicinal protons, respectively. Another downfield signal resonating at δ 5.52 showed connectivity in COSY spectrum to protons of the C-7 methylene group and was assigned to the C-6 vinylic proton. The methyl singlets at δ 1.15 and 1.27 were of Me-18 and Me-19, while those comparatively downfield at δ

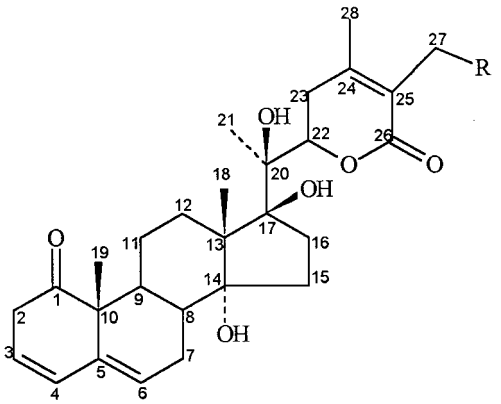
* Corresponding author. Tel.: +92-21-4968733; fax: +92-21-4963373.

E-mail address: amalik@amalik.hejric.edu (A. Malik).

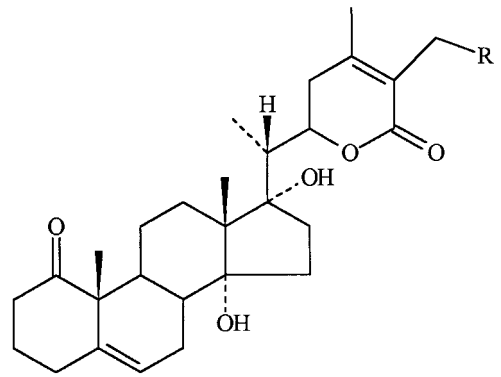
0031-9422/99/$ - see front matter
PII: S0031-9422(99)00345-3

1.41 and 2.01 could be assigned to Me-21 and one vinylic methyl of δ-lactone moiety. The absence of the other vinyl methyl and the appearance of two AB doublets at δ 4.20 and δ 4.11 which moved downfield to δ 4.45 and δ 4.32 in the corresponding monoacetate **1a**, suggested the presence of hydroxymethylene at either C-24 or C-25. Its location at C-25 was confirmed by HMBC experiment which revealed a ^2J correlation of oxymethylene protons (δ 4.20, 4.11) to C-25 (δ 125.2) and ^3J correlation to C-24 (δ 154.8) and C-26 (δ 166.5). The oxymethine proton resonating at δ 4.62 showed one-bond heteronuclear connectivity to a carbon at δ 80.1 in the HMQC spectrum of **1** and ^2J couplings to carbons at δ 32.5 (C-23) and 79.3 (C-20) and ^3J couplings to carbons at δ 88.6 (C-17) and 166.5 (C-26) in the HMBC, confirming its placement at C-22. It was assigned the α-orientation (22*R*) in analogy to commonly occurring withanolides. This assignment was confirmed through its multiplicity in the ^{1}H-NMR spectrum. It has been reported that when C-22 has the *S*-configuration, H-22 resonates as a broad singlet with $W_{1/2} \approx 5$ Hz while in the *R*-configuration it appears in the ^{1}H-NMR spectrum as a double doublet with two coupling constants characteristic for axial–axial and axial–equatorial interactions with H_2-23 (Vaina, Abdullaev & Abubakirrov, 1990). In the case of **1**, H-22 resonated as double doublet, revealing the *R* configuration at C-22. The occurrence of Me-21 as singlet and multiplicity of H-22 confirmed the presence of hydroxyl group at C-20. The remaining two oxygen atoms must be present as tertiary hydroxyls since five of the oxygen atoms have already been accounted and the monoacetate **1a** still showed hydroxyl absorption in its IR spectrum. One of these was assigned to C-14 because of its downfield shift in ^{13}C-NMR compared to a previously reported withanolide (Partha, Masao, Yasuo, Yuji, & Makoto, 1988) having similar rings A to C. The OH at C-14 was further confirmed by an HMBC experiment which showed a ^2J correlation of C-18 methyl protons (δ 1.15) to C-13 (δ 53.7) and ^3J to C-12 (δ 34.09), C-14 (δ 83.5) and C-17 (δ 88.6). The C-21 methyl protons (δ 1.41) also showed ^2J to C-20 (δ 79.3) and ^3J correlations to C-17 (δ 88.6) and C-22 (δ 80.1). It has been observed that 14β-OH does not cause shielding of C-12 (Glotter, Sahai, Kirson & Gottlieb, 1985), while 14α-OH shields C-7, C-9 and C-12 and deshields C-8 (Chen, Chen, Hsieh, Li & Wen, 1990). Thus, the 14-OH of **1** was assigned the α-orientation. The remaining hydroxyl was placed at C-17. In an HMBC experiment it showed ^3J correlation of C-17 at δ 88.6 with protons at δ 1.15 (Me-18) and δ 1.41 (Me-21). The β configuration of 17-OH could be deduced from the characteristic pyridine induced downfield shift for Me-18 as has been observed with other 17β-hydroxywithanolides (Bessalle & Lavie, 1992; Monteagudo, Burton, Gonzalez, Oberti & Gros, 1988). The ^{13}C-NMR spectrum showed signals for 28 carbons and their shift values were consistent with the above substitution pattern. Assignments of all functional groups were confirmed by HMQC and HMBC experiments and comparison with related withanolides (Ramaiah et al., 1984; Velde et al., 1983). Based on the above evidence, the structure 14α,17β,20,27-tetrahydroxy-1-oxo-(20*R*, 22*R*)-witha 3,5-24-trienolide was assigned to **1**.

The IR and UV spectra of Ajugin F [**2**] were similar to **1**. The high resolution FAB mass spectrum showed ion $[M + H]^+$ peak at *m/z* 473. 2858 corresponding to molecular formula $C_{28}H_{40}O_6$. The EI mass spectrum showed similar fragmentation pattern as **1** except the absence of peak at *m/z* 185 which was indicative of the absence of hydroxyl group at C-20 (Ramaiah et al., 1984). The ^{1}H- and ^{13}C-NMR spectra of **2** were very similar to **1** except in lacking one olefinic bond and a



1 R = OH
1a R = OAc



2 R = OH
2a R = OAc

tertiary hydroxyl group, respectively. Comparison of data with that of withametelin–H_2 having similar ring A, established the absorption pattern of both rings A and B (Shingu, Kajimoto & Nohara, 1987; Oshima, Bagchi & Hikino, 1987). The absence of hydroxyl group at C-20 was inferred from the mass spectrum and further confirmed by ^{1}H-NMR showing the signal of Me-21 as doublet at δ 1.10 while the multiplicity of H-22 also changed to doublet of double doublet at δ 4.18. The primary and one of the tertiary hydroxyl groups could be assigned to C-27 and C-14, based on HMBC correlations, acetylation to **2a** and chemical shift values of C-7, C-8, C-9 and C-12 which were similar to **1**. The remaining hydroxyl was located at C-17 on the basis of an HMBC experiment which showed ^3J correlations with δ 0.98 (Me-18) and 1.10 (Me-21). The α configuration of 17-OH could be deduced as, unlike **1**, downfield pyridine induced shift of Me-18 could not be observed (Bessalle & Lavie, 1992; Monteagudo et al., 1988) and also by comparing the data with reported withanolides having similar α-orientation (Nittala & Lavie, 1981). The structure 14α,17α,27-trihydroxy-1-oxo-(20*R*, 22*R*)-witha-5,24-dienolide was assigned to compound **2**.

3. Experimental

3.1. General

UV: MeOH on Hitachi U-3200 and IR KBr on Jasco-A-302 spectrometers. FAB–MS and HR–EIMS on Finnigan MAX 112 and JMS HX-110 spectrometers, respectively. ^{1}H- and ^{13}C-NMR spectra: $CDCl_3$ + few drops of CD_3OD on a Bruker AM-400 spectrometer operating at 400 MHz for ^{1}H and 125 MHz for ^{13}C nuclei. The 2D NMR experiments (COSY 45°, NOESY, HMBC and HMQC) were performed on the same instrument using the same solvent. The chemical shifts are in (δ) and coupling constants (J) are in Hz. Purity of the compounds was checked on silica gel GF254 coated cards.

3.2. Plant material

Ajuga parviflora (Labiatae), whole plant, was collected in July 1997 from Swat in NWFP province, Pakistan and identified by Dr. J. Shah. A voucher specimen (No PUH 14918) has been kept in the herbarium of Peshawar University.

3.3. Extraction and isolation

Whole plant material (20 kg) of *A. parviflora* was shade dried, ground and extracted thrice with MeOH at room temperature. The combined extracts were evaporated under reduced pressure to obtain a crude syrup which was defatted through repeated extraction with hexane. The defatted extract was subjected to vacuum liquid chromatography (VLC) ; silica gel 60 PF254 (1 kg), hexane–EtOAc and then EtOAc–MeOH in increasing order of polarity. The fractions obtained from EtOAc – MeOH (8.5 : 1.5) were combined and subjected to flash column chromatography; silica gel 200–440 mesh (400 g), EtOAc–MeOH in increasing order of polarity. The fractions obtained from EtOAc–MeOH (8 : 2) were subjected to low pressure liquid chromatography (MPLC); Lobar 9 Lichroprep Si 60 Merck column, EtOAc–MeOH (9 : 1). Final purification of the resulting fractions was achieved through preparative TLC on silica gel ($CHCl_3$ C_6H_6–MeOH–H_2O, 4 : 4 : 5 : 0.7) to obtain pure compounds **1** (20.2 mg) and **2** (24.2 mg), respectively.

3.4. Ajugin E (*1*)

White amorphous solid, $[\alpha]_D^{21}$: +125° (c = 0.058, MeOH); UV (MeOH): λ_{max} (ε) 223 (17950) nm; IR (KBr): ν_{max} = 3455, 1715, 1703 cm^{-1}; positive ion HR–FAB–MS: m/z 487.2697 $[M + H]^+$, $C_{28}H_{39}O_7$ requires M 487.2695; EI–MS; m/z (% rel. int.): 468 (7), 450 (12), 345 (8), 301 (28), 283 (11), 185 (41), 141 (100). ^{1}H-NMR (400 MHz, $CDCl_3$ + CD_3OD): δ = 1.15 (3H, *s*, 18-CH_3), 1.27 (3H, *s*, 19-CH_3), 1.41 (3H, *s*, 21-CH_3), 2.01 (3H, *s*, 28-CH_3), 4.20 (1H, *d*, J = 12 Hz, H-27), 4.11 (1H, *d*, J = 12 Hz, H′-27), 4.62 (1H, *dd*, J = 12.6 and 3.5 Hz, H-22), 5.52 (1H, br. d, J = 5.1 Hz, H-6), 5.76 (1H, *m*, H-3), 5.90 (1H, *dd*, J = 9.8 and 2.1 Hz, H-4), ^{13}C-NMR (125 MHz $CDCl_3$ + CD_3OD): δ = 19.4 (C-21), 20.1 (C-19), 20.5 (C-28), 20.6 (C-18), 20.8 (C-11), 25.7 (C-7), 31.7 (C-15), 32.5 (C-23), 34.09 (C-12), 34.1 (C-8), 35.6 (C-9) 37.9 (C-16), 39.6 (C-2), 53.7 (C-13), 53.9 (C-10), 56.1 (C-27), 79.3 (C-20), 80.1 (C-22), 83.5 (C-14), 88.6 (C-17), 121.2 (C-6), 125.2 (C-25), 127.4 (C-3), 129.3 (C-4), 140.2 (C-5), 154.8 (C-24), 166.5 (C-26), 210.5 (C-1).

3.5. Ajugin F (*2*)

White amorphous solid, $[\alpha]_D^{21}$ + 57° (c = 0.063, MeOH); UV (MeOH): λ_{max} (ε) 225 (18,000) nm; IR (KBr): ν_{max}: 3450, 1716, 1698 cm^{-1}; positive ion HR–FAB–MS; m/z 473.2858 $[M + H]^+$, $C_{28}H_{41}O_6$ requires M 473.2856; EI–MS: m/z (% rel. int.); 454 (9), 436 (8), 301 (22), 231 (22), 169 (35), 141 (100), 124 (18). ^{1}H-NMR (400 MHz, $CDCl_3$ + CD_3OD): δ; 0.98 (3H, *s*, 18-CH_3), 1.10 (3H, *d*, J = 6.3 Hz, 21-CH_3), 1.25 (3H, *s*, 19-CH_3), 1.96 (3H, *s*, 28-CH3), 4.20 (1H, *d*, J = 12 Hz, H-27), 4.11 (1H, *d*, J = 12 Hz, H′-27), 4.18 (1H, *ddd*, J = 12.5, 5.9 and 3.6 Hz, H-22), 5.59 (1H, br. d, J = 5.4 Hz, H-6), ^{13}C-NMR (125 MHz,

$CDCl_3$ + CD_3OD): δ = 9.8 (C-21), 17.4 (C-19), 18.7 (C-18), 20.5 (C-28), 20.9 (C-11), 25.7 (C-3), 26.2 (C-7), 28.9 (C-15), 31.6 (C-4), 31.7 (C-23), 33.8 (C-8), 34.0 (C-16), 34.5 (C-12), 35.0 (C-9), 37.6 (C-2), 41.5 (C-20), 50.4 (C-13), 51.1 (C-10), 56.2 (C-27), 78.9 (C-22), 84.6 (C-14), 86.3 (C-17), 121.1 (C-6) 122.8 (C-25) 140.4 (C-5) 155.0 (C-24), 166.6 (C-26), 215.8 (C-1).

3.6. Acetylation of **1** and **2**

A solution of the sample (10 mg) in pyridine (2 ml) and Ac_2O (2 ml) was stirred overnight at room temperature. Usual work up provided the corresponding acetyl derivatives **1a** and **2a**, respectively. Compound **1a** (10 mg), amorphous solid. UV (MeOH): λ_{max} (ε) 222 (19,000) nm; IR (KBr): $\nu_{max} = 3440$, 1712, 1702, 1695 cm^{-1}; EI–MS: *m/z* (% rel. int): 528 (M^+, 6), 468 (12), 450 (20), 308 (35), 124 (85). ^{1}H-NMR (400 MHz $CDCl_3$) δ = 1.14 (3H, *s*, 18-CH_3), 1.27 (3H, *s*, 19-CH_3), 1.42 (3H, *s*, 21-CH_3), 2.02 (3H, *s*, 28-CH_3), 2.40 (3H, *s*, OAc), 4.45 (1H, *d*, $J = 12$ Hz, HA-27), 4.32 (1H, *d*, $J = 12$ Hz HB-27), 5.50 (1H, br. d, $J = 5.2$ Hz, H-6), 5.74 (1H, *m*, H-3), 5.91 (1H, *dd*, $J = 9.8$, 2.5 Hz, H-4). Compound **2a** (11 mg), amorphous solid. UV (MeOH): λ_{max} (ε) 224 (18500) nm; IR (KBr): $\lambda_{max} = 3425$, 1713, 1702, 1690 cm^{-1}; EI–MS: *m/z* (% rel. int.) 514 (M^+, 4), 454 (12), 436 (18), 418 (10), 124 (12). ^{1}H-NMR (400 MHz, $CDCl_3$); $\delta = 0.98$ (3H, *s*, 18-CH_3), 1.10 (3H, *d*, $J = 6.3$ Hz, 21-CH_3), 1.25 (3H, *s*, 19-CH_3), 1.98 (3H, *s*, 28-CH_3), 2.20 (3H, *s*, OAc), 4.17 (1H, *ddd*, $J = 12.5$, 5.7, 3-6 Hz, H-22), 4.61 (1H, *d*, $J = 12$ Hz, HA-27), 4.58 (1H, *d*, $J = 12$ Hz HB-27), 5.57 (1H, br. d, $J = 5.5$ Hz H-6).

References

Bessalle, R., & Lavie, D. (1992). *Phytochemistry*, *31*, 3648.

Chen, C.-M., Chen, Z.-T., Hsieh, C.-H., Li, W.-S., & Wen, S.-Y. (1990). *Heterocycles*, *31*, 1371.

Glotter, E., Sahai, M., Kirson, I., & Gottlieb, H. E. (1985). *J. Chem. Soc. Perkintrans*, *1*, 2241.

Khan, P.M., Ahmad, S., Nawaz, H.R., & Malik, A. *J. Nat. Prod.*, in press.

Khan, P.M., Ahmad, S., Nawaz, H.R., & Malik, A. *Helv. Chim Acta*, in press.

Monteagudo, E. S., Burton, G., Gonzalez, C. M., Oberti, J. C., & Gros, E. G. (1988). *Phytochemistry*, *27*, 3925.

Nittala, S. S., & Lavie, D. (1981). *Phytochemistry*, *20*, 2741.

Oshima, Y., Bagchi, A., & Hikino, H. (1987). *Tetrahedron Letters*, *28*, 2025.

Partha, N., Masao, K., Yasuo, B., Yuji, M., & Makoto, S. (1988). *Bull. Chem. Soc*, *61*, 4479.

Pavia D. L., Lampman, G. M., Kriz, & G. S. (1979). *Introduction to spectroscopy*, Saunders College Publishing Co., Philadelphia, p. 41.

Ramaiah, P. A., Lavie, D., Budhiraja, R. D., Sudhir, S., & Garg, K. N. (1984). *Phytochemistry*, *23*, 143.

Rodriguez-Hahn, L., Esquivel, B., & Cardenas, J. (1994). *Prog. Chem. Org. Nat. Prod. Springer–Verlag*, *63*, 107.

Shingu, K., Kajimoto, T., & Nohara, T. (1987). *Chem. Pharm. Bull*, *35*, 4359.

Vaina, O. E., Abdullaev, N. D., & Abubakirrov, N. K. (1990). *Khim Prir. Soedin*, *3*, 366.

Velde, V. V., Lavie, D., Budhiraja, R. D., Sudhir, S., & Garg, K. N. (1983). *Phytochemistry*, *22*, 2253.

Wessner, M., Champion, B., Girault, J.-P., Kaouadji, N., Saidi, B., & Lafont, R. (1992). *Phytochemistry*, *31*, 3785.