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Megastigmane glycosides from seeds of *Trifolium alexandrinum*

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Abstract

Five megastigmane glycosides have been isolated from the seeds of *Trifolium alexandrinum* L., of which two are known compounds, while three are new compounds showing the presence of apiofuranosyl-(1→2)-glucopyranosyl residue as a sugar moiety. The structures of the isolated compounds were established on the basis of NMR and mass spectral data.

Keywords: *Trifolium alexandrinum*; Leguminosae; Megastigmane glycosides

1. Introduction

The seeds of *Trifolium alexandrinum* L. is popularly used in Egypt as an antidiabetic agent (Salah & El-Awady, 1961; Helmi, El-Mahdy, Ali, & Khayyal, 1969). Previously, oleanene-type triterpenoidal saponins (Mohamed, Ohtani, Kasai, & Yamasaki, 1995); quercetin, quercetrin and a new flavanonol derivative named trifolexin have been isolated from the seeds of this plant (Maatooq, 1997).

This paper deals with the isolation and structure elucidation of five megastigmane glycosides from the seeds of *Trifolium alexandrinum*, two of which are known glucoside derivatives (**1** and **2**) and they are reported for the first time from the family Leguminosae, while compounds **3**–**5** are new megastigmane glycosides displaying an apiofuranosyl-(1→2)-glucopyranosyl moiety.

2. Results and discussion

The seeds of *T. alexandrinum* were first defatted with hexane followed by extraction with methanol. The concentrated extract was diluted with water and partitioned between ethylacetate and water. The aqueous fraction was then applied on a column of Diaion HP 20 and eluted with water, 50% methanol and finally with methanol. The 50% methanolic eluate was subjected to repeated silica gel, RP-18 column chromatography and preparative HPLC using ODS column to give five compounds (**1**–**5**).

2.1. Structural formulae

Compound **1** had a molecular formula of $C_{19}H_{30}O_8$ as determined from the negative HR-FAB-mass spectrum and ^{13}C NMR spectral data. The ^{13}C and ^1H NMR spectra of **1** (Tables 1 and 2) together with DEPT mode measurement showed the presence of a β-D-glucopyranosyl moiety from the signals at δ_C 102.7 and δ_H 4.33 (1H, d, $J=7.8$ Hz). They showed also the existence of an aglycone with 13 carbon atoms which had a *trans*-disubstituted double bond, an α,β-unsaturated ketone, an oxygenated quaternary carbon, three tertiary methyls and a secondary methyl, suggesting a megastigmane (α-ionol) skeleton, which was confirmed by measurement of 2-D NMR including H–H COSY and HSQC spectral analyses. Enzymatic hydrolysis of **1** using β-glucosidase yielded D-glucose and an aglycone (**1a**) having identical physical data (m.p. and $[\alpha]_D$) as those previously reported for vomifoliol (Pousset & Poisson, 1969; Fukui, Koshimizu, Usuda, & Yamazaki, 1977; Okamura, Yagi, & Nishioka, 1981). From the aforementioned data compound **1** could be identified as (6*S*,7*E*,9*R*)-6,9-dihydroxymegastigma-4,7-dien-3-one-9-*O*-β-D-glucopyranoside or roseoside, which has previously been found in *Zizyphus jujuba* (Oka-

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Table 1
^{13}C NMR data of compounds **1**–**5** (100 MHz, CD_3OD)

C	1	2	3	4	5	5a[a]	5a[a]
1	42.4	37.1	42.4	37.1	37.5	36.3	36.2
2	50.7	48.3	50.7	48.4	48.4	47.7	47.5
3	201.1	202.0	201.2	202.0	202.6	198.4	198.6
4	127.2	126.2	127.2	126.1	125.6	125.2	125.2
5	167.2	165.8	167.3	165.9	170.3	165.3	165.2
6	80.0	56.8	80.0	56.8	52.6	51.1	51.0
7	131.5	128.8	131.5	128.8	26.9	25.9	26.3
8	135.3	138.2	135.3	138.2	38.1	37.2	38.9
9	77.3	77.0	76.9	76.6	78.2	75.7	67.6
10	21.2	21.0	21.2	21.0	25.2	24.4	25.7
11	23.4	27.6	23.4	27.6	27.8	27.1	27.1
12	24.7	28.1	24.7	28.1	29.3	28.7	28.6
13	19.5	23.8	19.5	23.8	19.8	19.5	19.5
Glc							
1′	102.7	102.5	101.3	100.9	100.7	100.3	
2′	75.2	75.3	79.3	79.3	79.0	79.0	
3′	78.0	78.0	77.9	77.8	78.0	77.7	
4′	71.6	71.5	71.7	71.6	72.2	72.1	
5′	78.1	78.1	78.0	77.9	78.0	77.9	
6′	62.8	62.7	62.8	62.7	63.1	62.9	
Api							
1″			110.8	110.8	110.7	110.4	
2″			78.6	78.6	78.8	78.2	
3″			80.6	80.6	80.9	80.9	
4″			66.1	66.1	66.6	66.7	
5″			75.4	75.3	75.7	75.7	

[a] These data were recorded in pyridine-d_5.

mura et al., 1981), *Vinca rosea* (Bhakuni, Joshi, Uprety, & Kapil, 1974), *Cydonia oblonga*, *Betula alba* (Tschesche, Ciper, & Harz, 1976), *Nicotiana tabacum* (Kodama, Fujimori, & Kato, 1981) and *Pinus sylvestris* (Andersson & Lundgren, 1988) and this is the first report on its occurrence in family Leguminosae.

The molecular formula of compound **2** was assigned from ^{13}C, DEPT ^{13}C NMR and negative HR-FAB-mass spectral data as $C_{19}H_{30}O_7$. The ^{13}C and ^{1}H NMR (Tables 1–2) of **2** showed a close similarity to **1** except for the signals at δ_C 56.8 with δ_H 2.66, 1H, d, $J=9.0$ Hz assigned for the methine carbon C-6 and other effects on C-1, C-5, C-7, C-12 and C-13 (see Table 1) due to the absence of a C-6 axial hydroxyl group compared to **1**. The results of 2-D H–H COSY and HSQC together with comparison the ^{13}C and ^{1}H NMR data of compound **2** with those reported before (Pabst, Barron, Semon, & Schrier, 1992) proved its structure as (6*R*,7*E*,9*R*)-9-hydroxymegastigma-4,7-dien-3-one-9-*O*-β-D-glucopyranoside which was isolated from raspberry fruit along with its (6*R*,7*E*,9*S*)-diastereomer (Pabst et al., 1992; Ito, Yasumoto, Kasai, & Yamasaki, 1994), and this is the first report on its isolation from the family Leguminosae. For further confirmation, compound **2** was subjected to enzymatic hydrolysis using β-glucosidase to afford D-glucose and the aglycone (6*R*,7*E*,9*R*)-9-hydroxymegastigma-4,7-dien-3-one (**2a**) with $[\alpha]_D$ +192° (Ref. Aasen, Kimland, & Enzell, 1973; Behr, Wahlberg, Nishida, & Enzell, 1978; +177°, for the naturally occurring (6*R*,7*E*,9*R*)-derivative and +69° for the synthetic (6*R*,7*E*,9*S*)-derivative (Behr et al., 1978).

The molecular formula of compound **3** was calculated as $C_{24}H_{38}O_{12}$ from ^{13}C NMR, DEPT ^{13}C NMR and negative HR-FAB-mass spectral data. Acid hydrolysis of **3** using 1 M HCl afforded **1a**, D-glucose and D-apiose identified by direct comparison with authentic samples. The negative FAB-mass spectral data of **3** supported the presence of an extra apiosyl moiety as compared with **1** by showing a quasi molecular anion at *m*/*z* 517 $[M-H]^-$ and a fragment peak at *m*/*z* 385 $[M-H-Api]^-$, indicating that apiose is the terminal sugar. Comparison the ^{13}C and ^{1}H NMR spectral data of **3** (Tables 1–2) with those of **1** revealed that **3** is an apiofuranosyl-derivative of

Table 2
^{1}H NMR data of compounds **1**–**5** (400 MHz, CD3OD)

H	1	2	3	4	5	5[a]
2a	2.50, 1H, d(16.5)	2.42, 1H, d(16.4)	2.52, 1H, d(16.5)	2.46, 1H, d(16.8)	2.49, 1H, d(17.1)	2.43, 1H, d(16.8)
2b	2.14, 1H, d(16.5)	2.03, 1H, d(16.4)	2.16, 1H, d(16.5)	2.08, 1H, d(16.8)	2.00, 1H, d(17.1)	2.02, 1H, d(16.8)
4	5.81, 1H, s	5.86, 1H, s	5.82, 1H, s	5.91, 1H, s	5.84, 1H, s	6.43, 1H, s
6		2.66, 1H, d(9.0)		2.71, 1H, d(9.0)	1.32, 1H, m	1.45, 1H, m
7	5.86, 1H, d(15.7)	5.65, 1H, dd(15.4, 9.0)	5.87, 1H, d(15.6)	5.67, 1H, dd(15.4, 9.0)	1.48, 2H, m	1.62, 2H, m
8	5.84, 1H, dd(15.7, 6.3)	5.76, 1H, dd(15.4, 6.4)	5.85, 1H, dd(15.6, 6.4)	5.80, 1H, dd(15.4, 6.3)	1.66, 2H, m	1.77, 2H, m
9	4.41, 1H, quint. (6.3)	4.39, 1H, m	4.42, 1H, m	4.42, 1H, m	3.26, 1H, m	3.85, 1H, m
10	1.27, 3H, d(6.3)	1.28, 3H, d(6.4)	1.27, 3H, d(6.4)	1.31, 3H, d(6.3)	1.21, 3H, d(6.1)	1.29, 3H, d(6.1)
11	1.02, 3H, s	0.99, 3H, s	1.01, 3H, s	1.04, 3H, s	1.04, 3H, s	0.92, 3H, s
12	1.03, 3H, s	1.02, 3H, s	1.02, 3H, s	1.06, 3H, s	1.13, 3H, s	1.04, 3H, s
13	1.91, 3H, s	1.92, 3H, s	1.92, 3H, s	1.97, 3H, d(1.2)	2.09, 3H, d(1.3)	1.85, 3H, s
1′	4.33, 1H, d(7.8)	4.34, 1H, d(7.9)	4.40, 1H, d(7.7)	4.45, 1H, d(7.8)	4.43, 1H, d(7.8)	4.85, 1H, d(7.6)
1″			5.39, 1H, d(1.4)	5.40, 1H, d(1.5)	5.40, 1H, d(1.2)	5.88, 1H, br.s

[a] Data was recorded in pyridine-d_5; *J* values in parentheses are recorded in Hz.

roseoside (**1**) as indicated from the signals at δ_C 110.8 (C-1″) with δ_H 5.39 (1H, d, $J=1.4$ Hz, H-1″) of the β-D-apiofuranosyl moiety (Otsuka et al., 1994) and the signals at δ_C 101.3 (C-1′) with δ_H 4.40 (1H, d, $J=7.7$ Hz, H-1′) of the β-D-glucopyranosyl unit. Their attachment together by a (1→2) interglycosidic linkage was deduced from the downfield shift of C-2′ of glucose to δ_C 79.3 with upfield shift of C-1′ to δ_C 101.3 when compared with the corresponding shifts of **1** at δ_C 75.2 and 102.7, respectively. Therefore, the new compound **3** was identified as (6*S*,7*E*,9*R*)-6,9-dihydroxymegastigma-4,7-dien-3-one-9-*O*-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside and named as trifostigmanoside I.

The elemental composition of compound **4** was determined as $C_{24}H_{38}O_{11}$ by negative HR-FAB-MS and ^{13}C NMR spectral analysis. The ^{13}C and ^{1}H NMR spectral data of **4** showed a close similarity to that of **2**, and inspection of the data (Tables 1–2) verified that the aglycone part was **2a** and the sugar part could be assigned as β-D-apiofuranosyl-(1→2)-β-D-glucopyranose, when compared with that of **3**. These assignments were achieved from the signals at δ_C 110.8 (C-1″) with δ_H 5.40 (1H, d, $J=1.5$ Hz, H-1″) and δ_C 100.9 (C-1′) with δ_H 4.45 (1H, d, $J=7.8$ Hz, H-1′) with downfield shift of C-2′ of glucose to δ_C 79.3 when compared with the corresponding shift of **2** at δ_C 75.3. For further confirmation, compound **4** was hydrolysed using 1 M HCl and the obtained products were identified as **2a**, D-glucose and D-apiose by direct comparison with authentic materials. Consequently, the new compound **4** was identified as (6*R*,7*E*,9*R*)-9-hydroxymegastigma-4,7-dien-3-one-9-*O*-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside and named as trifostigmanoside II.

Compound **5** was analyzed for $C_{24}H_{40}O_{11}$ (negative HR-FAB-MS and DEPT ^{13}C NMR. The ^{13}C and ^{1}H NMR (CD_3OD, Tabs. 1–2) of **5** exhibited signals corresponding to β-D-apiofuranosyl-(1→2)-β-D-glucopyranosyl moiety when compared with those of **3** and **4**. The remaining signals of the aglycone part indicated the presence of megastigmane skeleton with close similarity to **3** and **4** except for saturation of the double bond at C-7 to give two methylenes at δ_C 26.9 and 38.1 corresponding to C-7 and C-8, respectively with δ_H 1.48 and 1.66 (each 2H, m) for H-7 and H-8, respectively. Comparing the NMR data of **5** in pyridine-d_5 (Tables 1–2) with those reported earlier (Galbraith & Horn, 1972; Miyase, Ueno, Takizawa, Kobayashi, & Oguchi, 1988) identified the aglycone part as blumenol C. The acid hydrolysis of **5** using 1 M HCl afforded D-glucose and D-apiose in addition to blumenol C (**5a**) with $[\alpha]_D$ +110.3°, which was in a good agreement with the reported value (Ref. Galbraith & Horn, 1972; +112.5°). The NMR spectrum of **5a** in pyridine-d_5 (Table 1) showed that C-9 was shifted upfield to δ_C 67.6 in comparison with that of **5** at δ_C 75.7 (glycosylation shift value = −8.2) assuming the chirality at C-9 as *R*-configuration (Otsuka et al., 1994). In the NOESY experiment of **5**, correlation peaks between H-6 and H-12 and H-13 were observed indicating α-axial orientation of H-6. The chirality at C-6 was established to be *R*-configuration as shown by molecular models which was also in accordance with the naturally occurring blumenol C (Galbraith & Horn, 1972). From the above mentioned data, the new compound **5** could be characterized as (6*R*,9*R*)-9-hydroxymegastigma-4-en-3-one-9-*O*-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside and named as trifostigmanoside III.

3. Experimental

Mp. uncorr.; ^{1}H and ^{13}C NMR (TMS as int. standard): 400 and 100 MHz, respectively. FAB-MS: direct inlet method at an ionizing voltage of 70 eV. HPLC: D-ODS-5 column (20 mm I.D. × 25 cm) with differential refractometer as detector; flow rate of mobile phase 6 ml/min, injection volume 0.8–1.0 ml. CC: Kieselgel 60 (70–230 mesh, Merck) and Diaion HP 20 (Mitsubishi). TLC: silica gel 60 precoated plates, F-244 and HPTLC using RP-18 precoated plates, F-244 s (Merck).

3.1. Plant material

Seeds of *T. alexandrinum* L. (local name: Bersim) was collected from Assiut, Egypt, in August 1996. The plant was authenticated by Professor A. Fayed, from the Department of Botany, Faculty of Science, Assiut University, Assiut, Egypt. A voucher specimen is deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Assiut University, Assiut, Egypt.

3.2. Extraction and isolation of compounds (*1*–*5*)

Air dried and powdered seeds (1 kg) were defatted (×3) by maceration with hexane, followed by extraction with MeOH under reflux conditions. The combined methanolic extracts were filtered, evapd. under red. pres. The residue suspended in H_2O and partitioned with EtOAc. The aq. fr. was applied to a column of Diaion HP 20 and eluted with H_2O, 50% MeOH and finally with MeOH. The 50% MeOH eluate (6.0 g) was chromatographed by silica gel CC using EtOAc–MeOH–H_2O (8:2:1 and 6:2:1 successively) to give 6 frs. Fr. 1 (520 mg) was sepd. by a column of reversed phase RP-18 using 50% MeOH to afford **1** (114 mg) and **2** (11 mg). Fr. 3 (240 mg) was chromatographed on a column of RP-18 using 40% MeOH followed by prep. ODS HPLC using 42.5% MeOH to give **3** (9 mg), **4** (13 mg) and **5** (25 mg).

3.3. Compound *1*

Amorphous powder, $[\alpha]^{22}_D$ +111.2° (MeOH; *c* 1.33). HR-FAB-MS (negative) *m/z*: 385.1884 $[M-H]^-$

$C_{19}H_{29}O_8$, req. 385.1862, 223 [M–H–glc]$^-$. ^{13}C and ^1H NMR (CD_3OD, Tables 1–2).

3.4. Compound 2

Amorphous powder, $[\alpha]^{22}_D$ +95.2° (MeOH; *c* 1.33). HR-FAB-MS (negative) *m/z*: 369.1929 [M–H]$^-$ $C_{19}H_{29}O_7$, req. 369.1913, 207 [M–H–glc]$^-$. ^{13}C and ^1H NMR (CD_3OD, Tables 1–2).

3.5. Enzymatic hydrolysis of 1 and 2

To a solution of **1** (40 mg) in 5 ml acetate buffer (pH 5.0) was added 50 mg of β-glucosidase, and the mixture was incubated with stirring at 37°C for 48 h and the mixture was extracted with Et_2O (3 × 10 ml). The combined ethereal layers were evapd. and the residue was purified by repeated crystallization from C_6H_6–EtOAc mixture to afford 11 mg of the aglycone vomifoliol (**1a**), mp 111–113°C, $[\alpha]^{22}_D$ +231.7° ($CHCl_3$; *c* 0.73). In the aq. phase D-glucose was identified by TLC comparison with an authentic sample. By the same procedure, 6 mg of **2** was hydrolyzed to give D-glucose and the aglycone **2a** (3.2 mg) as an oily liquid, $[\alpha]^{22}_D$ +192.4° ($CHCl_3$; *c* 0.21).

3.6. Compound 3

Amorphous powder, $[\alpha]^{22}_D$ +52.9° (MeOH; *c* 0.62). HR-FAB-MS (negative) *m/z*: 517.2291 [M–H]$^-$ $C_{24}H_{37}O_{12}$, req. 517.2284, 385 [M–H–Api]$^-$, 223 [M–H–Api–glc]$^-$. ^{13}C and ^1H NMR (CD_3OD, Tables 1–2).

3.7. Compound 4

Amorphous powder, $[\alpha]^{22}_D$ +16.3° (MeOH; *c* 0.80). HR-FAB-MS (negative) *m/z* : 501.2351 [M–H]$^-$ $C_{24}H_{37}O_{11}$, req. 517.2336, 369 [M–H–Api]$^-$, 207 [M–H–Api–glc]$^-$. ^{13}C and ^1H NMR (CD_3OD, Tables 1–2).

3.8. Compound 5

Amorphous powder, $[\alpha]^{22}_D$ +77.1° (MeOH; *c* 0.87). HR-FAB-MS (negative) *m/z*: 503.2499 [M–H]$^-$ $C_{24}H_{39}O_{11}$, req. 503.2492, 371 [M–H–Api]$^-$, 209 [M–H–Api–glc]$^-$. ^{13}C and ^1H NMR (CD_3OD, Tables 1–2).

3.9. Acid hydrolysis of compounds 3–5

Compound **3** (5 mg) in 1 M HCl (10 ml) was refluxed at 80°C for 3 h. The reaction mixture was diluted with H_2O and then extracted with Et_2O, from the ethereal layer the aglycone **1a** (2.2 mg) was obtained. In the aq. phase D-apiose and D-glucose were identified by TLC comparison with authentic substances. By the same procedure, 6 mg of **4** was hydrolyzed to give D-apiose, D-glucose and the aglycone **2a** (3 mg). Compund **5** (12 mg) was also hydrolyzed by the same way to afford D-apiose, D-glucose and 6.4 mg of the aglycone blumenol C (**5a**) as an oily liquid, $[\alpha]^{22}_D$ +110.3° ($CHCl_3$; *c* 0.42), ^{13}C NMR Table 1.

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References

Aasen, A. J., Kimland, B., & Enzell, C. R.(1973). *Acta Chem. Scand.*, *27*, 2107.
Andersson, R., & Lundgren, L. N.(1988). *Phytochemistry*, *27*, 559.
Behr, D., Wahlberg, I., Nishida, T., & Enzell, C. R.(1978). *Acta Chem. Scand. B*, *32*, 391.
Bhakuni, D. S., Joshi, P. P., Uprety, H., & Kapil, R. S.(1974). *Phytochemistry*, *13*, 2541.
Fukui, H., Koshimizu, K., Usuda, S., & Yamazaki, Y.(1977). *Agric. Biol. Chem.*, *41*, 175.
Galbraith, M. N., & Horn, D. H. S.(1972). *J.C.S. Chem. Comm.*, *3*, 113.
Helmi, R., El-Mahdy, S. A., Ali, H., & Khayyal, M. A. H.(1969). *J. Egypt. Med. Assoc.*, *52*, 538.
Ito, A., Yasumoto, K., Kasai, R., & Yamasaki, K.(1994). *Phytochemistry*, *36*, 1465.
Kodama, H., Fujimori, T., & Kato, K.(1981). *Biol. Chem.*, *45*, 941.
Maatooq, G. T.(1997). *Mans. J. Pharm. Sci.*, *13*, 70.
Miyase, T., Ueno, A., Takizawa, N., Kobayashi, H., & Oguchi, H.(1988). *Chem. Pharm. Bull.*, *36*, 2475.
Mohamed, K. M., Ohtani, K., Kasai, R., & Yamasaki, K.(1995). *Phytochemistry*, *40*, 1237.
Okamura, N., Yagi, A., & Nishioka, I.(1981). *Chem. Pharm. Bull.*, *29*, 3507.
Otsuka, H., Kamada, K., Ogimi, C., Hirata, E., Takushi, A., & Takeda, Y.(1994). *Phytochemistry*, *35*, 1331.
Pabst, A., Barron, D., Semon, E., & Schrier, P.(1992). *Phytochemistry*, *31*, 1649.
Pousset, J. L., & Poisson, J.(1969). *Tetrahedron Lett.*, *15*, 1173.
Salah, M. K., & El-Awady, M. M.(1961). *J. Pharmaceut. Sci. U.A.R.*, *2*, 117.
Tschesche, R., Ciper, F., & Harz, A.(1976). *Phytochemistry*, *15*, 1990.