Antibacterial Diterpenoids from *Astragalus brachystachys*

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Antibacterial bioassay guided fractionation of acetone extracts of *Astragalus brachystachys* resulted in isolation of sclareol and two related labdane-type diterpenoids, 14*R*-epoxysclareol and 6β-hydroxysclareol. The antibacterial activity of the isolated compounds was measured and it was deduced that the epoxidation at the double bond of sclareol or hydroxylation at C-6 decreased the activity of the resulting compounds. Salvigenin (5-hydroxy-4′,6,7-trimethoxyflavone) was also separated from this plant for the first time.

Introduction

The genus *Astragalus* with about 800 species is one of the most abundant genera of the flowering plants in Iran (Mozaffarian, 1998). Some of these plants contain aliphatic nitro compounds as the poisonous components which caused moderate to heavy loss of cattle and sheep on the western rangeland of the United States (Cronin *et al.*, 1981; Williams and James, 1975). Several species of the genus *Astragalus* from Iran have been studied on their nitro and sterol contents (Ebrahimzadeh *et al.*, 1999 and 2001). Antibacterial properties of an acetone extract of *Astragalus brachystachys* prompted us to investigate its constituents by TLC bioautography guided fractionation and purification. Identification of sclareol (**1**) as the most active constituent followed by 14*R*-epoxysclareol (**2**) and 6β-hydroxysclareol (**3**) was the result of this investigation. Salvigenin was also separated from the acetone extract as the major flavonoid. To the best of our knowledge the plant *Astragalus brachystachys* has not been studied chemically or biologically so far. The oxidized sclareol derivatives were reported as the microbial transformation products of sclareol, amongst them epoxysclareol (Farooq and Tahara, 2000; Hanson *et al.*, 1994) and 6α-hydroxysclareol (Aranda *et al.*, 1991a and 1991b) are the minor constituents. Two diastereomers of epoxysclareol were obtained from epoxidation of sclareol using *m*-chloroperbenzoic acid (MCPBA) (Mela *et al.*, 1998). The antibacterial activity of sclareol was determined previously against several pathogenic bacteria (Ulubelen *et al.*, 1985).

This paper reports the isolation, structure elucidation and antibacterial activities of 14*R*-epoxysclareol (**2**) as a new natural product from a plant and 6β-hydroxysclareol (**3**) as a novel compound.

Results and Discussion

Silica gel column and flash column chromatography and preparative TLC using different solvent systems, guided by antibacterial TLC autobiography (Hamburger and Cordell, 1987) of the acetone extract of *Astragalus brachystachys* resulted in isolation of three labdane-type diterpenoids, sclareol (**1**), 14*R*-epoxysclareol (**2**) and 6β-hydroxysclareol (**3**). Salvigenin was isolated and identified as the major flavonoid in the extract.

Structure elucidation of the constituents

Sclareol (Torrenegra *et al.*, 1992) and salvigenin (Chari *et al.*, 1981) were identified by comparison of their spectral data with the literature values.

The HREI mass spectrum of **2** showed a molecular ion peak at 324.2671 consistent with a molecular formula $C_{20}H_{36}O_3$ (*calcd.* 324.2664). In the

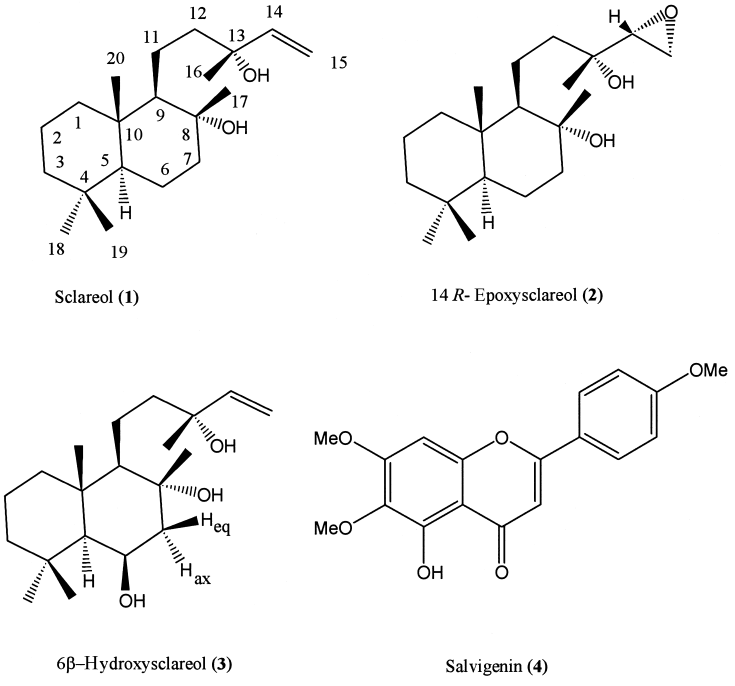

Fig. 1. Isolated compounds from *Astragalus brachystachys*.

IR spectrum the absorption bands at ν_{max} 1264, 1190, 868 and 755 cm^{-1} besides the characteristic band of the hydroxyl group at 3358 cm^{-1} was consistence with the presence of the epoxide functional group in the molecule.

In the ^1H NMR spectrum of **2**, the signals at δ 2.88 (*dd*, *J* = 2.7, 3.7 Hz), 2.70 (*t*, *J* = 4.2 Hz), and 2.81 (*dd*, *J* = 2.7, 4.9 Hz) were considered as the signals for the protons of a terminal epoxide moiety in the molecule. The other signals of **2** were similar to those recorded for **1**. For instance despite the oxidation of the double bond no drastic changes observed for methyl signals, Me-16 (δ 1.26), Me-17 (1.13), Me-18 (0.76), Me-19 (0.84) and Me-20 (0.76) in comparison for the corresponding methyl signals of sclareol at δ 1.27, 1.16, 0.78, 0.86 and 0.78. In the ^{13}C NMR spectrum of compound **2**, twenty carbon signals were observed. Replacement of the carbon signals of the double bond of sclareol by a methine carbon at δ 58.4 and a terminal methylene at δ 44.3 was evidence for epoxidation of the C-14/C-15 double bond in **2**. The ^{13}C NMR signals of C-12, C-13 and C-16 of **2** were shifted up-field in comparison with those of **1** at δ 41.2, 70.0 and 24.9. $\Delta\delta = \delta\mathbf{2} - \delta\mathbf{1}$ for the above signals are −3.7, −3.7 and −2.4 respectively. The stereochemistry at C-14 was determined using NOE difference spectroscopy. Irradiation at δ 2.88 enhanced the signals of Me-16 (1.95%), H_a-15, and H-12 at δ 1.54 (3.6%). On the other hand Me-16 on irradiation of its signal showed correlation with a series of signals at δ 1.5–1.7 (3.6%) which is consistence with H_β-1, and a proton of H-11. Me-16 also showed correlations with H-14 (1.34%) and H_b-15 (0.5%) which suggest the stereochemistry at C-14 as *R*. Considering the absolute stereochemistry of sclareol as *R* at C-13, the configuration of C-14 must be *R* in **2** (Figure 2).

The molecular ion of compound **3** was determined by FAB negative and ESI mass spectroscopies. An ion at 323.2602 in the HR-FAB mass

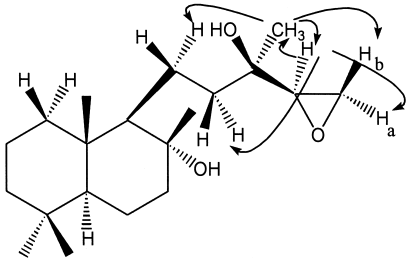

Fig. 2. The NOE correlations for **2**.

Table I. ^{1}H NMR and ^{13}C NMR spectral data for **1** and **2** (in $CDCl_3$) and **3** (in CD_3OD).

Position	**1**, δ_C	**2**, δ_C	**3**, δ_C	**1**, δ_H, *J* (Hz)	**2**, δ_H, *J* (Hz)	**3**, δ_H, *J* (Hz)
1	39.7	39.7	43.4	0.95, 1.6*	0.92 *m*, 1.62 *m*	0.94 *m*, 1.65 *m*
2	18.5	18.2	19.7	1.4, 1.6*	1.38 *m*, 1.58 *m*	1.42 *m*
3	42.0	42.0	45.4	1.13, 1.4*	1.38 *m*	1.30 *m*, 1.12 *m*
4	33.3	33.2	35.1	–	–	–
5	56.1	56.1	58.4	0.91*	0.90 *m*	0.94 *m*
6	20.6	20.4	68.8	1.3, 1.6*	1.62 *m*	4.41 *br d* (2.7)
7	44.4	44.1	52.5	1.84 *ddd* (3.0, 3.0, 11.8, H-7eq)	1.81 *ddd* (2.9, 2.9, 9.4 H-7eq), 1.38 *m*	1.98 *br dd* (2.7, 14.0, H-7eq), 1.59 *br dd* (3.0, 14.0 H-7ax)
8	74.8	74.5	74.6	–	–	–
9	61.6	62.0	63.3	1.11, 1.5*	1.06 *m*	1.10 *m*
10	39.3	39.2	40.8	–	–	–
11	19.2	18.5	20.5	1.3, 1.65*	1.38 *m*, 1.58 *m*	1.54 *m*
12	44.9	41.2	46.7	1.65*	1.54 *m*	1.74 *m*, 1.54 *m*
13	73.7	70.0	74.4	–	–	–
14	145.8	58.4	146.6	5.92 *dd* (10.7, 17.3)	2.88 *dd* (2.7, 3.7)	5.92 *dd* (10.8, 17.5)
15	111.2	44.3	111.6	5.21 *br d*, (17.5, H-15$_{trans}$), 5.02 *br d* (10.7, H-15$_{cis}$)	2.70 *t* (4.2, H_a), 2.81 *dd* (2.7, 4.9, H_b)	5.19 *dd*, (1.5, 17.5, H-15$_{trans}$), 5.00 *dd* (1.5, 10.8, H-15$_{cis}$)
16	27.3	24.9	27.3	1.27 *s*	1.26 *s*	1.23 *s*
17	24.3	24.2	25.3	1.16 *s*	1.13 s	1.34 *s*
18	33.5	33.4	33.7	0.78 *s*	0.76 *s*	0.96 *s*
19	21.6	21.5	24.1	0.86 *s*	0.84 *s*	1.18 *s*
20	15.5	15.4	17.2	078 *s*	0.76 *s*	1.17 *s*

* The values are reported from literature (Torrenegra *et al.*, 1992).

spectrum represented the [M-H]-, $C_{20}H_{35}O_3$ (*calcd.* 323.2586) for **3**. On the other hand an ion at 347 (100%) was observed in ESI spectrum which was assigned as $[M+Na]^+$. The IR spectrum established the presence of a hydroxyl group with absorption at 3393 cm^{-1}. The ^{1}H NMR spectrum (in CD_3OD, Table I) was similar to that recorded for **1** except the peaks for a signal at δ 4.41 (*brd*, J = 2.7 Hz) for a hydrogen geminal to hydroxyl. Incorporation of this hydroxyl in the molecule resulted in changes in the chemical shifts of the adjacent protons' signals especially those for the methyls. The ^{13}C NMR spectrum showed 20 signals for **3** which were classified to methyl (five), methylene (seven), methine (four) and quaternary (four) carbons using DEPT experiment. The assignment of the protons and carbons was confirmed using interpretation of HMQC and HMBC spectra. The position of the hydroxyl group was determined using HMBC experiment. The cross peaks between H-7eq with the carbon signals of C-5, C-6, C-8, C-9, and C-17 and H-7_{ax} and C-8 and C-17 and those between H-6 (δ 4.41) and C-8 and C-10 were established the position of the hydroxyl at C-6. The small coupling constant for H-6 (*d*, J = 2.7 Hz) together with downfield shifted signals of three methyl groups 1,3-diaxially substituted, relative to 6β-hydroxyl: Me-17, Me-19 and Me-20 at δ 1.34, 1.18 and 1.17 respectively, suggested the orientation of C-6 hydroxyl as axial. In the NOESY spectrum the cross peaks between H-7_{eq} and Me-17 and between H-6 and H-5, H-7_{ax} were consistence with our conclusion about the stereochemistry at C-6 hydroxy group. The stereochemistry at C-13 was decided according to its chemical shift in ^{13}C NMR and comparison to those recorded for the relevant carbon in sclareol (Torrenegra *et al.*, 1992) and 6α-hydroxysclareol (Aranda *et al.*, 1991a, 1991b). The signal of C-16 was shifted to downfield in case of episclareol (13*S*) at δ 29.1 (in $CDCl_3$) in comparison with 26.8 for sclareol (Torrenegra *et al.*, 1992). But in the spectra of sclareol (in $CDCl_3$) and 6α-hydroxysclareol (in CD_3OD) the signals were recorded at δ 27.3 and 27.5 respectively (Aranda *et al.*, 1991a, 1991b). The chemical shifts of other signals of 6α-hydroxysclareol in the side chain were matched with those recorded for **3**, so the configuration at C-13 must be *R*. The stereochemistry at C-6 was confirmed by ^{13}C NMR spectral data. The up-field

Table II. Antibacterial activities of sclareol (**1**) and its derivatives (**2** and **3**).

A: on TLC bioautography test.

Test Organism	**1**	**2**	**3**	PCP
Bacillus subtilis	100 / 30 a 50 / 25 20 / 14	100 / 20 50 / 15 20 / 13	100 / 11 50 / 8 20 / 8	2.3 / 19 1.6 / 17 1 / 15

B: by paper disk diffusion method.

Test Organism	**1**	**2**	**3**	PCP
Bacillus subtilis	200 / 18 b 160 / 11 120 / 10 80 / 9 40 / 8	200 / 0 160 / 0 120 / 0 80 / 0 40 / 0	200 / 0 160 / 0 120 / 0 80 / 0 40 / 0	20 / 32 16 / 31 12 / 27 8 / 23 4 / 0

[a] The amount (μg) of the compound charged on TLC plate/the inhibition zone diameter (millimeter).
[b] The amount (μg) of the compound charged on each paper disk/the inhibition clear zone diameter (millimeter).

shifts observed for the signals of C-5 and C-7 at δ 58.4 and 52.5 for **3** while the values 62.1 and 55.5 were recorded for the mentioned signals in the 6α-hydroxy derivative.

The results of the antibacterial bioassays

Table II (A and B) show the results of antibacterial activities of sclareol and its derivatives on TLC bioautography and disk diffusion tests (against *Bacillus subtilis*) respectively. In both of them, TLC bioautography and disk diffusion methods, sclareol found to be more active with an inhibition zone of 14 and 8 mm i.d. with 20 and 40 μg amount of charged compound on TLC plates and paper disks, respectively. The test with the same amounts for **2** and **3** on TLC showed 13 and 8 mm i.d. inhibition zones respectively while an inhibition zone of 15 mm i.d. was determined with 1 μg of pentachlorophenol as the positive control. The order of decreasing of the activity: sclareol > 14*R*-epoxysclareol > 6β-hydroxysclareol is consistence with decreasing of the polarities of the compounds. Biotransformation of sclareol with fungi to its oxidized derivatives may be the result of the detoxification of this compound by these microorganisms (Farooq and Tahara, 2000; Hanson *et al.*, 1994; Aranda *et al.*, 1991a, 1991b).

Experimental

General procedure

^{13}C NMR spectra (broad band and DEPT experiments) were measured on a JEOL, JNM-EX270 instrument at 67.5 MHz. ^{1}H NMR, ^{1}H-^{1}H COSY, ROESY, HMQC and HMBC experiments were performed on a 500 MHz Bruker AMX500. MS spectra were recorded on a JEOL JMS-SX102A spectrometer. Optical rotations were determined on a JASCO DIP-370 digital polarimeter. The UV and IR spectra were recorded on a Hitachi model U-3210 spectrophotometer and a Perkin Elmer System 2000 FT-IR spectrometer, respectively. Analytical TLC experiments were performed on Merck silica gel 60 F_{254}, DIOL F_{254} HPTLC and RP-18 WF_{254} HPTLC pre-coated glass plates. Column chromatographies were performed, on silica gel (100–210 μm) and (40–63 μm), LiChroprep Diol (40–63 μm) and Cosmosil $75C_{18}$-OPN as stationary phases.

Plant material

All parts of the plant, *Astragalus brachystachys* DC. were collected around Kerman, Iran in April 2001. The plant was identified by Dr. Valiollah Mozaffarian at Botany Research Division, Research Institute of Forests and Rangelands, Tehran-Iran. A voucher specimen is kept with one of us (P. A. A.)

Extraction of the plant material and purification of the compounds

The shade dried plant material (481 g) after grinding was extracted with acetone (8 lit.) for one week. The extraction was repeated twice and the acetone extracts after evaporation under reduced pressure (22 g) were subjected to silica gel (100–210 mesh, 500 g) column chromatography. The col-

umn eluted with *n*-hexane with increasing of polarity up to ethyl acetate followed by methanol. The fractions eluted with about 40% ethyl acetate in hexane up to pure ethyl acetate were monitored by TLC bioautography to detect antibacterial components. To purify sclareol the fractions (1.7 g) eluted with 40–50% ethyl acetate in hexane were subjected to flash column chromatography over silica gel (40–63 μm, 45 g). The column was eluted with hexane-chloroform (1:1) with increasing of polarity to pure chloroform followed by 4% methanol in chloroform. Compound **1** (256 mg) was obtained in fractions eluted with nearly pure chloroform.

The fractions (248 mg) eluted with about 60–80% chloroform in hexane contained a major UV quenching constituent detected on TLC plate. A portion of the mentioned fraction (48 mg) was subjected to preparative silica gel TLC using chloroform-acetone (99:1 v/v) to purify salvigenin (25 mg).

The later fractions (2 g) of the first column chromatography eluted with ethyl acetate and methanol (prominent in ethyl acetate) were loaded on a flash silica column (70 g). The column was eluted with chloroform with increasing of the polarity (1% gradient) by methanol. Twenty-three fractions (each 100 ml) were collected from the column. Fraction 16 (441 mg) was loaded on a column containing Cosmosil 75C_{18}-OPN (30 g) and eluted with methanol-water (1:1) followed by increasing the percentage of methanol up to pure methanol. The fractions eluted with *ca.* 90% methanol in water were combined (184 mg) and purified by column chromatography over LiChroprep Diol (40–63 μm, 10 g) using hexane-chloroform (1:1) and increasing the polarity to pure chloroform. Compound **2** (22 mg) was obtained in the fractions collected with 60–80% hexane chloroform.

The fractions prominent in chloroform which showed antibacterial activity on TLC were added to each other (68 mg) and loaded on a column, containing Cosmosil 75C_{18}-OPN (3 g) and eluted with methanol-water (1:1) followed by increasing the percentage of methanol up to pure methanol. Compound **3** (31 mg) was purified from fractions predominant in methanol.

TLC bioautography

The acetone crude extract of the plant material was analyzed by silica gel TLC plates (ethyl acetate-hexane = 1:1 v/v, as the mobile phase). A suspension of the gram positive bacteria, *Bacillus subtilis* (IFO 12113) and the gram negative bacteria, *Escherichia coli* (IFO 3301) in nutrient broth were sprayed on the developed TLC plates. The TLC plates were incubated overnight at 25 °C. The TLC plates then sprayed with INT (*p*-iodonitrotetrazolium violet) solution (0.5 g/100 ml H_2O) and incubated to visualize the purple color. Three white spots, representing the antibacterial constituents, two in the middle and one on the base line of the chromatogram were observed in case of *Bacillus subtilis.* After purification of antibacterial components, **1**, **2** and **3**, they were subjected to antibacterial tests using different amounts. Compounds **1**, **2**, and **3** exhibited antibacterial activity against *Bacillus subtilis*, but were inactive against the gram negative bacteria at the presented amount in Table II (A). Pentachlorophenol was used as the antibacterial control in the appropriate amounts shown.

Paper disk diffusion method

A range of test compounds **1**, **2**, and **3** (40–200 μg) were dissolved in acetone and applied onto paper disk of Φ 8 mm. The dried papers were placed on agar seeded with *Bacillus subtilis* in a Petri dish. The Petri dishes were stand overnight at 4 °C, so that the metabolites could diffuse in the medium. The plates were incubated at 37 °C for 18 h. The antibacterial activity was determined by measuring the diameters of the clean inhibitory zone around each paper disk. As shown in Table II (B) pentachlorophenol (PCP) was used as the positive control (Ishikawa *et al.*, 2001).

Sclareol (**1**)

Oil; $[\alpha]_D^{22}$ −6.4 ($CHCl_3$, *c* 0.742) lit.: $[\alpha]_D^{25}$ −2.82 ($CHCl_3$, *c* 1.45) (Kouzi and McChesney, 1990). For ^{13}C and ^{1}H NMR data see Table I.

14R-Epoxysclareol (**2**)

White precipitates, m. p. 131–133 °C (methanol) lit.: 128–129 °C (Farooq and Tahara, 2000). $[\alpha]_D^{22}$ −8.3 ($CHCl_3$, *c* 1.72) lit.: $[\alpha]_D^{25}$ −9 (MeOH, *c* 0.01) (Farooq and Tahara, 2000). IR ν_{max}^{NaCl} cm^{-1}: 3387, 2925, 2868, 2359, 1459, 1388, 1264, 1190, 1137, 1084, 938, 868, 755. HREI-MS: *m/z* 324.2671 $[M]^+$ ($C_{20}H_{36}O_3$ *calcd.* 324.2664); EI-MS

m/z (*rel. int.* %): 324([M]$^+$, 5), 306 ([M-H_2O]$^+$, 10), 294 (15), 263 (23), 245 (83), 205 (18), 191 (40), 177 (100), 123 (48), 69 (56). For ^{13}C and ^{1}H NMR data see Table I.

6β-Hydroxysclareol (**3**)

White precipitates (methanol); $[\alpha]_D^{22}$ −11.5 (MeOH, *c* 4.50); IR ν_{max}^{NaCl} cm^{-1}: 3393, 2923, 1546, 1458, 1386, 1304, 1092, 995, 915, 755. HR-FAB-MS: negative *m/z* 323.2602 [M-1]$^-$ ($C_{20}H_{35}O_3$ *calcd.* 323.2586); EI-MS *m/z* (*rel. int.* %): 306 ([M-H_2O]$^+$, 1), 292 (1.8), 220 (21), 191 (20), 71 (100). For ^{13}C and ^{1}H NMR data see Table I.

Salvigenin (*5-hydroxy-4,6,7-trimethoxyflavone,* **4**)

EI-MS *m/z* (*rel. int.* %): 328 ([M]$^+$, 100), 313 ([M-CH_3]$^+$, 78), 299 (17), 285 (19), 282 (19), 153 (17). UV λ_{max} (MeOH): 330, 277, 215 nm. ^{1}H NMR ($CDCl_3$, 270 MHz) δ: 12.76 (1H, *s*), 7.84 (1H, *d*, *J* = 8.8 Hz), 7.02 (1H, *d*, *J* = 8.8 Hz), 6.54 (1H, *s*), 6.58 (1H, *s*), 3.97, 3.92, 3.89 (each 3H, *s*); ^{13}C NMR (CDCL3, 67.5 MHz) δ: 182.5, 163.8, 162.4, 158.6, 152.9, 127.9, 123.5, 114.4, 104.1, 90.5, 60.9, 56.3, 55.6.

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