Amanicadol, a Pimarane-type Diterpene from *Phlomis amanica* Vierch.

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Fractionation of the methanol extract of *Phlomis amanica* resulted in the isolation of a new pimarane type diterpene, amanicadol (**1**), together with the known glycosides lamiide, verbascoside (= acteoside), syringaresinol-4-*O*-β-glucoside, liriodendrin, syringin, and a caffeic acid ester, chlorogenic acid. The structure of the new compound was established on the basis of extensive 1D and 2D NMR spectroscopic data interpretation. Molecular modeling studies on **1** were conducted and showed that it exhibited low conformational flexibility. Additionally, NMR chemical shifts were calculated for **1** *in vacuo*, and calculated values were in very close agreement with those found experimentally.

Key words: Phlomis amanica, Lamiaceae, Amanicadol, Diterpene, Pimarane

Introduction

The genus *Phlomis* (Lamiaceae) is represented by 34 species in the flora of Turkey [1]. Some *Phlomis* species are used as tonics and stimulants in Anatolian folk medicine [2]. A few members of the genus are used for their antiinflammatory, wound healing, and pain relief properties in Chinese medicine [3, 4].

Our previous phytochemical studies on Turkish *Phlomis* species were focused on glycosidic compounds. However, in a continuation of our phytochemical investigations on the same species, we have now isolated a new pimarane type diterpene, amanicadol (**1**) from the *n*-hexane extract of *Phlomis amanica*, an endemic species. Chromatographic separations on the *n*-BuOH extract of the title plant afforded the known glycosides lamiide, verbascoside (= acteoside), syringaresinol-4-*O*-β-glucoside, liriodendrin, syringin as well as a caffeic acid ester, chlorogenic acid.

Results and Discussion

Compound **1** was obtained as an amorphous powder. The molecular formula was established as $C_{20}H_{32}O$ on the basis of a HRESIMS molecular ion peak at $m/z = 299$ $[M+H]^+$, and the analysis of its ^1H and ^{13}C NMR spectroscopic data (Table 1). The IR spectrum of **1** displayed absorption bands typical for hydroxyl groups and double bonds. The combined analysis of the ^{13}C NMR and DEPT spectra revealed the presence of 20 carbon signals assigned to four methyls, seven methylenes, five methines (one tertiary alcohol and two olefinic carbon atoms), and four quaternary carbon atoms. These data, together with the molecular composition, suggested that **1** possessed 5 degrees of unsaturation. The presence of two π-bonds further indicated that the compound was a tricyclic diterpene. The ^1H NMR spectrum showed signals due to four tertiary methyl groups at $\delta = 1.01$, 0.91, 0.87 and 0.85, three vinylic protons from a monosubstituted double bond at $\delta = 5.90$, 4.93, and 5.02 [ABX pattern, $J_{AB} = 1.3$ Hz, $J_{AX} = 10.5$ Hz (*cis*-coupling), and $J_{BX} = 17.5$ Hz (*trans*-coupling)], a geminal proton of a secondary hydroxyl group at $\delta = 4.05$ (dt, $J = 4.8$ and 6.6 Hz), and a singlet resonance at $\delta = 5.30$ in addition to the proton signals assigned to the rings A, B and C. A detailed analysis of DQF COSY data

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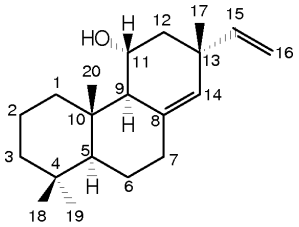

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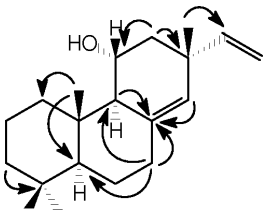

Fig. 1. Selected HMBC correlations of **1** (H → C).

revealed the identification of three spin systems arising from the rings A, B and C, H_2-1/H_2-2/H_2-3, H-5/H_2-6/H_2-7, and H-9/H-11/H_2-12, respectively. In the ^{13}C NMR spectrum the carbon resonances appearing at $\delta = 149.1$ (C-15) and 110.6 (C-16) supported the presence of an exocyclic vinyl group. This proposal was also confirmed by the long range ^{1}H-^{13}C NMR (Fig. 1) correlation between the methyl signal at $\delta = 1.01$ (H-17) and the carbon resonance at $\delta = 149.1$ (C-15) as well as HMBC correlations between the vinylic protons ($\delta = 4.93$ and 5.02) and the quaternary signal at $\delta = 37.5$ (C-13). An olefinic methine carbon signal was observed at $\delta = 127.4$ (C-14), which showed an HMQC correlation with the proton resonance at $\delta = 5.30$. In the HMBC spectrum this olefinic hydrogen coupled to C-8 ($\delta = 136.5$) indicating the presence of a trisubstituted double bond in the ring C. All of these findings indicated that compound **1** possessed a pimara-8(14),15-diene skeleton [5–7]. The signal at $\delta = 4.05$ (dt, $J = 4.8$ and 6.6 Hz), observed in the spin system of ring C, was attributed to a geminal proton signal of a secondary hydroxyl group. The location of the secondary hydroxyl group was determined as C-11 by analysing the DQF-COSY and HMBC spectra. The relative stereochemistry of **1** was established by NOE DIFF and NOESY experiments with mixing times of 400, 800 and 1200 msec. In all NOESY experiments NOE correlations were observed between Me-18 and Me-20, Me-20 and H-11, and also between H-11 and Me-17 which was suggestive of a relative β-axial orientation of H-11, revealing OH-11 to be α and equatorial [8]. Additionally, an NOE correlation observed between H-9 and H-5 indicated that both hydrogen atoms were in a relative α-axial orientation. This confirmed the *trans* A:B ring junction stereochemistry of **1**. Compound **1** was assigned as *rel*-(5*S*, 9*R*, 10*S*, 11*R*, 13*R*)-11-α-hydroxypimara-8(14), 15 diene.

Table 1. ^{13}C ($CDCl_3$, 100.0 MHz) and ^{1}H ($CDCl_3$, 400.0 Hz) NMR data for **1**. Values in parenthesis are calculated chemical shifts.

C	DEPT	δ_C (ppm)	δ_H (ppm)	J (Hz)
1	CH_2	40.2 (41.67)	1.33 (1.54) d	4.0
			1.89 (1.56) dd	12.7/1.4
2	CH_2	19.1 (21.81)	1.57 (1.59) t	3.1
3	CH_2	42.1 (41.89)	1.24 (1.31) d	4.4
			1.41 (1.41) m	–
4	C	33.4 (37.18)		
5	CH	54.9 (55.82)	1.13 (1.21) dd	12.5/2.6
6	CH_2	23.1 (25.60)	1.65 (1.57)*	–
			1.30 (1.52) dd	12.9/4.4
7	CH_2	36.3 (37.64)	2.34 (2.16)m	–
			2.10 (2.12) m	–
8	C	136.5 (134.55)		
9	CH	60.0 (62.1)	1.77 (1.89) d	4.8
10	C	39.1 (44.4)		
11	CH	66.2 (66.4)	4.05 (4.27) dt	4.8/6.6
12	CH_2	43.5 (43.16)	1.65 (1.59)*	–
13	C	37.5 (42.45)		
14	CH	127.4 (126.92)	5.30 (5.45) s	–
15	CH	149.1 (143.92)	5.90 (6.05) dd	17.5/10.5
16	CH_2	110.6 (106.02)	4.93 (5.09) dd	10.5/1.3
			5.02 (5.15) dd	17.5/1.3
17	CH_3	27.0 (23.23)	1.01 (1.14) s	–
18	CH_3	22.1 (22.66)	0.87 (0.86) s	–
19	CH_3	33.8 (33.87)	0.91 (0.92) s	–
20	CH_3	15.9 (16.96)	0.85 (1.00) s	–

* Unclear due to signal overlapping.

The 3D structure of **1** was built using the Maestro modelling software package and the conformational search was performed using Macromodel [9, 10]. It was found that **1** exibited low conformational flexibility. Furthermore, the geometry was optimized using DFT calculations utilizing the B3LYP/6-31** basis set [11]. The distances between different groups of hydrogen atoms were consistent with observed NOE signals (Fig. 2). The NMR chemical shifts were calculated for **1** *in vacuo*, which gave some remarkable agreements with experimentally found values (Table 1). Structures and chemical shifts for other possible stereoisomers were also calculated, but those could not explain the presence of the NOE signals, and the calculated values of chemical shifts did not agree so well with experimentally determined chemical shifts. This has suggested that our stereospecific assignments a correct. A literature survey revealed that **1** was a

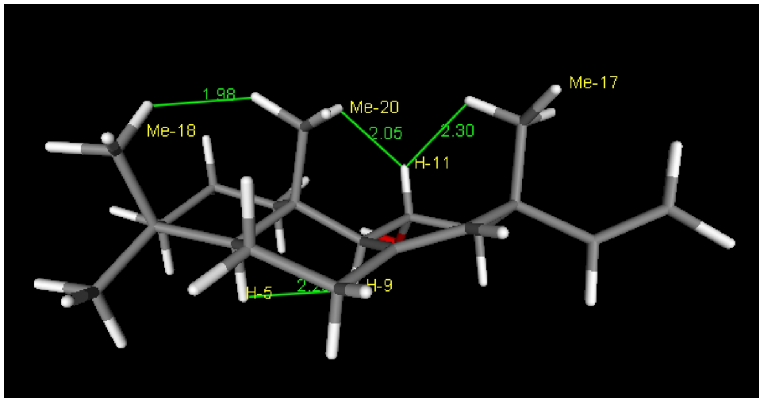

Fig. 2. 3D structure of **1**. Labels depict hydrogen atoms that exibit NOE correlations used for the assignment of the stereochemistry. (Distances in Å).

new compound from nature and the trivial name amanicadol is proposed.

The structures of the known compounds lamiide (**2**) [12], verbascoside (**3**) [13], syringaresinol-4-*O*-β-glucoside (**4**) [14], liriodendrin (**5**) [15], syringin (**6**) [16, 17], and chlorogenic acid (**7**) [18] were identified by their physical and spectroscopic (^{1}H NMR, ^{13}C NMR, DEPT, 2D NMR and MS) data and by comparing the data obtained with those published in the literature.

Conclusion

Phlomis species are generally known to contain glycosidic secondary metabolites such as iridoids, phenylethanoids, flavonoids, and lignans. Up to date only some labdane-type diterpenes have been reported from the genus [19, 20]. This is the first report of the isolation and characterization of a pimarane-type diterpene from a member of the genus *Phlomis*.

Experimental Section

General experimental procedures

Optical rotations were recorded on a Rudolph Autopol IV polarimeter. UV (CH_2Cl_2) spectra were recorded on a Shimadzu UV-160A spectrophotometer. FTIR (KBr) spectra were determined on a Perkin-Elmer 2000 FTIR spectrophotometer. NMR measurements in $CDCl_3$ were performed on a JEOL JNM-A400 FT-NMR (^{1}H: 400 and ^{13}C: 100 MHz) and a Bruker AVANCE 500 spectrometer (^{1}H: 500 and ^{13}C: 125 MHz). Chemical shifts were given in ppm with tetramethylsilane as an internal standard.

Accurate mass measurements were determined on a Micromass Q-TOF Ultima Global Tandem Mass Spectrometer. The sample was run under electrospray ionization mode using 50% acetonitrile in water and 0.1% formic acid as a solvent. The instrument was calibrated before analysis using the ions produced from [Glu]-fibrinopeptide B as an internal standard, $[M+2H]^{2+} = 785.8426$.

For open column chromatography (CC) Kieselgel 60 (0.063 – 0.200 mm, Merck) and Sephadex LH-20 (Fluka) were used. MPLC was performed on a Büchi (2.5×46 cm) glass column packed with LiChroprep RP-18 (Merck) (40 – 63 μm) using a Büchi B-684 pump. VLC separation was realized on a small glass column (5.2×10 cm) packed with LiChroprep RP-18 (Merck) (40 – 63 μm). TLC was carried out on pre-coated Kieselgel 60 F_{254} aluminium sheets (Merck). Compounds were detected by UV fluorescence and by spraying with 1% vanillin/H_2SO_4, followed by heating at 100 °C for 1 – 2 min.

Plant material

Phlomis amanica Vierch. was collected from Arsuz (Hatay), in the vicinity of Kale village, 151 m, Southwest Anatolia, Turkey. Voucher specimens have been deposited in the Herbarium of the Department of Biology, Faculty of Science, Hacettepe University (AAD 10654).

Extraction and isolation

Air-dried and powdered aerial parts of *P. amanica* (450 g), were extracted with MeOH (3×2 L) at 40 °C. The concentrated methanolic extract was suspended in H_2O (100 mL) and partitioned between *n*-hexane (4×100 mL), $CHCl_3$ (4×100 mL), and *n*-BuOH (4×100 mL). 8.8 g of the *n*-hexane extract was separated on a silica gel column (40×100 cm) with a solvent gradient of CH_2Cl_2-MeOH ($100:0 \rightarrow 50:50$), to afford six main fractions (Frs. I – VI, 200 mL, each). Fr. II (305 mg) was further rechromatographed by Sephadex LH-20, eluting with cyclohexane-acetone-dichloromethane-methanol (1 : 1 : 1 : 2). The elution volume of each fraction was (Frs II_{1-10}) 200 mL each. Fr. II_{5-6} (180 mg) was applied to a silica gel column eluting with a gradient of

n-hexane-EtOAc (99 : 1 → 95 : 5, 25 mL each) to give **1** (30 mg).

The *n*-BuOH extract (30 g) of *P. amanica* was first chromatographed on a polyamide column with MeOH-H_2O (0 : 100 → 100 : 0) mixtures to give Frs. A (1.49 g), B (8.5 g), C (1.2 g), D (3.3 g), and E (3.76 g). Fr. B was applied to VLC using a RP-18 column with MeOH-H_2O (0 : 100 → 45 : 55) mixtures to afford **2** (5 mg). Fr. B_{13-16} (134 mg), obtained from VLC, were subjected to repeated column chromatographic separations on Sephadex LH-20, RP-18 and silica gel to yield **5** (4 mg) and **6** (6 mg). Fr. C (1.2 g) was first subjected to MPLC and eluted with MeOH-H_2O (0 : 100 → 100 : 0) to give 120 fractions (Frs. C_{1-120}). Fr. C_{98-108} (200 mg) was rechromatographed by silica gel column chromatography, followed by VLC to afford **4** (4 mg) and **7** (12 mg). Fr. E (3.76 g) was applied to VLC using MeOH-H_2O (0 : 100 → 45 : 55) mixtures to yield **3** (15 mg).

*Amanicadol (**1**)*

Amorphous powder; $[\alpha]_D^{20} - 0.029°$ ($CHCl_3$, *c* 1) UV/vis (CH_2Cl_2): $\lambda_{max}(\lg \varepsilon_{max}) = 244$ nm (3.99). IR [KBr]: ν_{max} = 3400, 3080, 1634, 1595. ^{1}H and ^{13}C NMR data are given in Table 1, HRESIMS, *m/z* = 289.2522 $[M+H]^+$ (calcd. for $C_{20}H_{32}O$ 289.2526).

Molecular modelling

The 3D structure of **1** was generated using the Maestro 7.5 package [9]. The conformational searching was performed using the Monte Carlo method [21] and OPLS-AA force [22] included in Macromodel 9.1 [10]. The most stable conformers were further optimized using Jaguar 6.5 [23] at the B3LYP/6-31** level of theory. ^{1}H and ^{13}C shielding constants were computed by *pseudo* spectral methods [24]. The theoretical chemical shifts were calculated taking into account TMS as the reference compound whose shielding constants were calculated under the same conditions.

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