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Abietane diterpenoids and triterpenoic acids from *Salvia cilicica* and their antileishmanial activities

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Abstract

Bioguided-fractionation of an acetone extract of the roots of *Salvia cilicica* (Lamiaceae) led to isolation of two new diterpenes, 7-hydroxy-12-methoxy-20-nor-abieta-1,5(10),7,9,12-pentaen-6,14-dione and abieta-8,12-dien-11,14-dione (12-deoxy-royleanone), together with oleanolic acid, ursolic acid, ferruginol, inuroyoleanol and cryptanol. Their structures were determined spectroscopically, which included HREIMS and 2D NMR spectroscopic analysis. The new abietane derivatives showed appreciable in vitro antileishmanial activity against intracellular amastigote forms of both *Leishmania donovani* (IC_{50} values of 170 and 120 nM, respectively) and *Leishmania major* (IC_{50} values of 290 and 180 nM, respectively). The triterpenoic acids were found to be potently active against amastigote (IC_{50} values of 7–120 nM) and moderately active against promastigote stages (IC_{50} values of 51–137 nM) of the two *Leishmania* species.

Keywords: *Salvia cilicia*; Lamiaceae; Diterpenoids; Triterpenoids; Antileishmanial

1. Introduction

Leishmaniasis is a major worldwide health problem causing morbidity and mortality with many clinical manifestations in humans (Ashford et al., 1992). Pentavalent antimonials, the first choice for chemotherapy, have variable efficacy, severe side effects and require long courses of administration. In addition, the development of therapy-resistant parasite strains and drug availability are becoming a major problem (Croft, 1997). Given the limitations of the current treatments, there is an urgent need for the development of new therapeutics.

The genus *Salvia* (Lamiaceae) consists worldwide of ca 900 species. In Turkey, about 90 species are growing naturally, almost half being endemic (Davis, 1982). *Salvia* species are used in folk medicines for the treatment of a variety of diseases, including infectious conditions. Studies on their chemical constituents have revealed the presence of a large variety of terpenoids (Ulubelen and Topcu, 1998) and polyphenols (Lu and Foo, 2002). A number of di- and some triterpenoids have been found to possess powerful antileishmanial activity (Corona et al., 2000). Continuing our research program to identify novel antileishmanial compounds and having in mind the abundant occurrence of terpenoid constituents in *Salvia* species, root extracts of *Salvia cilicica* Boiss and Kotschy were tested in vitro against extracellular promastigote and intracellular amastigote forms of *Leishmania donovani*, causative agent of visceral leishmaniasis, and *L. major*, causative agent of cutaneous leishmaniasis. No chemical investigation on *S. cilicica*, an endemic species of Turkey, has so far been published.

This paper reports on the isolation and structure elucidation of two new abietanes, associated with five known compounds from this plant source and their in vitro antileishmanial activity.

2. Results and discussion

Crude lipophilic fractions obtained by column chromatography on silica gel from the parent acetone extract of the roots of *Salvia cilicica* showed moderate in vitro antileishmanial activity against *Leishmania donovani* and *L. major*. Bioguided fractionation of the active fractions eluted with petroleum ether–ethyl acetate gradient systems led to isolation of two new diterpenoids.

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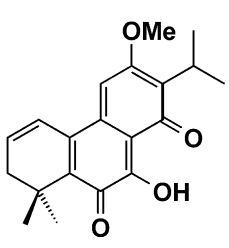
Initial identification of compounds **1** and **2** as diterpenoids clearly followed from their visualisation on silica plates upon treatment with $Sb(Cl)_3$ and $Ce(SO_4)_2$.

Compound **1** was isolated as an amorphous solid and established to have a molecular formula of $C_{20}H_{22}O_4$ by HR-EIMS (M^+ at m/z 326.1513; calc. 326.1531). Its IR spectrum showed a chelated carbonyl absorption at 1628 cm^{-1} and the UV absorptions at λ_{max} 269 and 476 nm were suggestive of a highly conjugated system. From the ^1H and ^{13}C NMR spectra signals for an abietane skeleton could be concluded. Unambiguous proton and carbon signal assignments were achieved on the basis of 2D experiments (COSY, HETCOR, HMQC). The ^{13}C NMR spectrum obtained with the aid of DEPT spectral analysis exhibited 20 resonances, for 5× CH_3, 1× CH_2, 4× CH and 10× C. A diagnostic feature in the ^1H NMR spectrum of **1** was the presence of a one-proton septet at δ 3.32 (H-15) for the methine proton of an isopropyl group. The corresponding cross-peaks of this signal with both the methyl doublets H_3-16 and H_3-17 appeared in the ^1H–^1H-COSY. Besides these two *sec*-methyls, well-separated signals of two *tert*-methyls, H_3-18 (δ 1.19) and H_3-19 (δ 1.21), appeared in the aliphatic region and a resonance of a methoxy group was displayed at δ 3.97 (CH_3O-12). The long-range correlations observed between H-15 (δ 3.32) and carbons C-13 (δ_C 136.4), C-12 (δ_C 158.8), C-14 (δ_C 189.9) as well as CH_3-16 and CH_3-17 (δ_C 19.3) confirmed the location of the isopropyl group at C-13. The position of an enolic hydroxyl group (δ 13.13), assigned to C-7, was established through long-range correlations with both C-7 (δ_C 160.6) and C-8 (δ_C 111.8) in the HMQC experiment (Table 1). Attachment of the methoxy group to C-12 clearly followed from the connectivity of the methoxyl protons (δ 3.97) with C-12 (δ_C 158.8). Additional significant associations observed in the HETCOR and HMQC experiments were the connectivities of the methylene protons at C-3 with both C-2 and C-4, and couplings between C-10 and H-1 as well as H-11 (Table 1). Notably, H-11 showed a $^4J_{CH}$ correlation with C-7, being in perfect agreement with the functionalization of the abietane skeleton established on the basis of HETCOR and HMQC correlations. Thus, compound **1** was identified as 7-hydroxy-12-methoxy-20-nor-abieta-1,5(10),7,9,12-pentaen-6,14-dione, a new natural diterpenoid.



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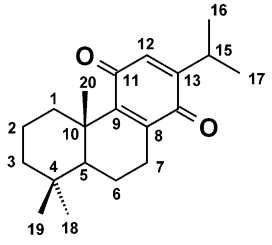

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Compound **2** was isolated as an amorphous powder, having the elemental composition of $C_{20}H_{28}O_2$ as concluded from the HR-EIMS. UV (269 nm) and IR spectra (1648, 1598 cm^{-1}) suggested the presence of a *para*-benzoquinone element (Shishido et al., 1994). Analysis of the ^1H NMR data (Table 1) of **2** again revealed the presence of an isopropyl group in the abietane skeleton. Inspection of ^1H–^{13}C long-range correlations established the location of this substituent at C-13 through the couplings of the diagnostic methine signal of H-15 (δ2.98) to C-14 (δ_C 188.1). Supporting evidence for this placement was available from connectivities between H-12 (δ 6.31) to C-15 (δ_C 26.3), C-14 (δ_C 188.1), C-11 (δ_C 188.0) and C-13 (δ_C 152.8) (Table 1). Taking into account the remaining signals due to three *tert*-methyls, five methylene groups, one methine proton and seven quaternary carbons as well as the results of 2D experiments (COSY, HETCOR, HMQC), the ^1H and ^{13}C NMR data of**2** were reminiscent of those of royleanone (Edwards et al., 1962), except for the lack of the HO-12 signal. Compound **2** was, therefore, defined as abieta-8,12-dien-11,14-dione (12-deoxyroyleanone). This is the first isolation of **2** from a natural source.

The known compounds **3** and **4** were identified as ursolic acid (**3**) and oleanolic acid (**4**) by direct comparison with authentic samples, and compounds **5**–**7** as ferruginol (**5**) (Nakanishi et al., 1983), inuroyleanol (**6**) (Bhat et al., 1975) and cryptanol (**7**) (Ulubelen et al., 1987) on the basis of their spectral and physical data. Whereas **3** and **4** were obtained from subfractions exhibiting interesting antileishmanial activities, compounds **5**–**7** were isolated from some inactive fractions.

2.1. Leishmanicidal activity

Subsequent in vitro studies examined the activity of compounds **1**–**4** against both promastigote and amastigote forms of *Leishmania donovani* and *L. major*. As a parameter for antileishmanial activity, the IC_{50} value, i.e. the sample concentration causing 50% reduction in survival/viability of the parasites, was used. With IC_{50} values ranging from 121 to 288 nM, the diterpenoids **1** and **2** were only moderately effective against the amastigote forms of the two *Leishmania* species residing within macrophage-like RAW 264.7 cells. Sodium stibogluconat (Pentostam®), used as a reference exhibited in the same conditions IC_{50} values in the range of 10 nM, being considerably more active than the test compounds (Table 2). In contrast, none of these abietanes showed antiparasitic activity when tested against the promastigote forms (IC_{50} > 300 nM). Interestingly, ursolic (**3**) and oleanolic acid (**4**) showed antileishmanial effects against both the extra- and intracellular forms of the parasites. Particularly **3** exhibited activities against intracellular amastigotes of the same range as the therapy standard Pentostam (IC_{50} of 7.0 and 12.7 nM compared

Table 1
^1H, ^{13}C NMR data and HMQC-couplings of diterpenoids **1** and **2** ($CDCl_3$, *J* values (Hz) are given in parentheses; ^1H: 400 MHz, ^{13}C: 100.6 MHz)

No	**1**			**2**		
	δ_H	δ_C	HMQC	δ_H	δ_C	HMQC
1	7.65 (1H, *dt* 9.9, 1.8)	123.4	H_2-3	1.09 (1H, *m*)[a] 2.74 (1H, *dt*, 12.5, 3.5)	36.5	Me-20
2	6.15 (1H, *dt* 9.9, 4.6)	130.6	H-1, H_2-3	1.53 (1H, *dt*, 14.2, 3.6) 1.73 (1H, *dt*, 14.2, 3.5)	19.0	
3	2.18 (2H, *dd* 4.6, 1.8)	36.6	H-1	1.18 (1H, *m*) 1.48(1H, *m*)	41.4	H_b-1, Me-18, Me-19
4		33.9	H_2-3		33.6	Me-18, Me-19, H_b-3
5		154.7	H-1, H_2-3, Me-18, Me-19	1.08 (1H, *m*) [a]	51.6	H_a-6, H_b-6, H_b-7, Me-18, Me-19, Me-20
6		182.6		1.42 (1H, *m*) 1.86 (1H, *br dd*, 13.5, 7.4)	17.4	
7	13.13 (1H, *s*, OH)	160.6	7-OH	2.30 (1H, *ddd*, 20.0, 11.5, 7.4) 2.69 (1H, *br dd*, 20.0, 5.0)	26.0	H_a-6, H_b-6, H-5
8		111.8	H-11, 7-OH		142.7	H_b-6, H_a-7, H_b-7
9		133.8			150.9	H_a-7, H_b-7, H-12, Me-20
10		126.6	H-1, H-11		38.5	H_a-6, H_b-6, Me-20
11	7.11 (1H, *s*)	119.4			188.0	H-12
12		158.8	CH_3O-12, H-15	6.31 (1H, *d*, 1.0)	132.0	
13		136.4	H-15, Me-16, Me-17		152.8	H-15, Me-16, Me-17
14		189.9	H-15		188.1	H-12
15	3.32 (1H, *septet*, 7.0)	23.2		2.98 (1H, *septet*, *d*, 7.0, 1.0)	26.3	Me-16, Me-17, H-12
16	1.20 (3H, *d*, 7.0)	19.3	H-15	1.09 (3H, *d*, 7.0)	21.4	
17	1.20 (3H, *d*, 7.0)	19.3	H-15	1.10 (3H, *d*, 7.0)	21.4	
18	1.19 (3H, *s*,)	30.9		0.93 (3H, *s*)	33.5	
19	1.21 (3H, *s*)	27.4	H_2-3	0.90 (3H, *s*)	21.8	
20	–	–		1.28 (3H, *s*)	20.2	
12-OMe	3.97 (3H, *s*)	59.8				

[a] Overlapping with Me-16 and Me-17.

to 9.8 and 10.6 nM, respectively). Except for **3** (IC_{50} 15.5 nM) as the most powerful candidate, none of the compounds showed significant cytotoxicity (IC_{50} >100 nM) when tested against non-parasitised macrophage-like RAW 264.7 cells alone as a mammalian host cell control. The use of compounds **1**, **2** and **4** appears limited by an unfavourable antileishmanial/cytotoxic ratio. Appropriate formulations, which overcome some of the toxic limitations should therefore prove useful as has been demonstrated for amphotericin B (Croft, 1997).

Table 2
In vitro antileishmanial activities (IC_{50} nM) of compounds **1**–**4** against promastigotes (PM) and amastigotes (AM) of *L. donovani* and *L. major*, and their toxicity for RAW 264.7 host cells

Compound	*L. donovani*		*L. major*		Toxicity for RAW cells
	PM	AM	PM	AM	
1	> 300.0	170.0	>.300.0	287.4	>.300.0
2	> 300.0	121.0	> 300.0	182.3	191.0
3	91.0	12.7	51.3	7.0	15.6
4	91.0	62.9	137.0	119.9	132.7
Pentostam	3.5	10.6	3.6	9.8	n.d.

n.d., Not determined

3. Experimental

3.1. General

UV spectra were recorded on a Shimadzu UV-240 spectrophotometer and IR spectra were measured on a Perkin Elmer 1420 ratio recording infrared spectrophotometer. ^1H NMR (400 MHz) and ^{13}C NMR (100.6 MHz) spectra were obtained using a Bruker AC-400 instrument. HMQC experiments were recorded on a Bruker AMX-400 spectrometer, optimized for $^{2-3}J_{H/C}$ = 10 Hz. EIMS and HR-EIMS were acquired with a Varian MAT CH_7A and a Finnigan MAT 711 spectrometer, respectively. Prep. TLC plates were used without activation (Merck, Kieselgel 60 F_{254}). Compounds were visualized by spraying with $Sb(Cl)_3$ and $Ce(SO_4)_2$.

3.2. Plant material

The roots of *Salvia cilicica* were collected at Adana-Pozantý (Turkey) in September 1997 at an altitude of about 1850 m. The plant material was identified by Professor Dr Neriman Özhatay (University of Istanbul), and a voucher specimen (ISTE 74581) is deposited in

the herbarium of the Faculty of Pharmacy, University of Istanbul.

3.3. Extraction and isolation

Dried and powdered roots of *S. cilicica* (1 kg) were exhaustively extracted with Me_2CO in a Soxhlet apparatus. The combined extracts were filtered and evaporated to dryness in vacuo. The residue (10 g) was subsequently chromatographed on a silica gel G 60 column (Merck, 6 × 130 cm) using a petroleum ether- EtOAc gradient system (1:0 → 0:1). The fraction 200–1600 ml eluted with petroleum ether–EtOAc (98:2) was subjected to TLC separation (Merck, Kieselgel 60 F_{254}, 0.5 mm) with petroleum ether–toluene (1:1) to yield pure compound **1**. Similar purification of fraction 1620–2800 ml using petroleum ether-toluene (4:1) as developing system, afforded compound **2**.

Ursolic acid (**3**) and oleanolic acid (**4**) were obtained from active subfractions of the latter portion, while the known diterpenoids ferruginol (**5**), inuroyleanol (**6**) and cryptanol (**7**) represented metabolites from inactive eluates.

3.4. 7-Hydroxy-12-methoxy-20-nor-abieta-1,5(10),7,9,12-pentaen-6,14-dione (*1*)

R_f 0.3; UV ($CHCl_3$) λ_{max} nm (log ϵ): 245 (2.61), 467 (1.79). UV (MeOH) λ_{max} nm 269, 300 sh, 476. IR ν_{max} (KBr) cm^{-1}: 2969, 1461, 1260, 1089, 1021, 798. HR-EIMS *m/z* 326.1513 (M^+, calc. for $C_{20}H_{22}O_4$ 326.1531); EI-MS *m/z* (rel. int.): 326 $[M]^+$ (100), 311 $[M–Me]^+$ (61), 283 $[M–C_3H_7]^+$. ^1H and ^{13}C NMR data: see Table 1.

3.5. Abieta-8,12-dien-11,14-dione (12-deoxyroyleanone) (*2*)

R_f 0.4; $[\alpha]_D^{20}$ −60.0° ($CHCl_3$, c 0.05). UV ($CHCl_3$) λ_{max} nm (log ϵ): 261 (3.21). UV λ_{max} (MeOH) nm: 269. IR ν_{max} (KBr) cm^{-1}: 2923, 2853, 1731, 1648, 1598, 1462. HR-EIMS *m/z* 300.2075 (M^+, calc. for $C_{20}H_{28}O_2$); EI-MS *m/z* (rel. int.): 300 $[M]^+$ (85), 285 $[M–Me]^+$(30), 257 $[M–C_3H_7]^+$(15), 243 (60), 204 (100). ^1H and ^{13}C NMR data: see Table 1.

3.6. Assays for leishmanicidal and cytotoxic activity

Assays for extra- and intracellular leishmanicidal activity including cytotoxicity testing against host cells were performed according to Kolodziej et al. (2001).

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