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Guaianolide sesquiterpenes from *Pulicaria crispa* (Forssk.) Oliv.

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ABSTRACT

A phytochemical study of the asteraceous herb *Pulicaria crispa* (Forssk.) Oliv. resulted in the characterisation of three guaianolide sesquiterpenes, 2α,4α-dihydroxy-7αH,8αH,10αH-guaia-1(5),11(13)-dien-8β,12-olide (**1**), 1α,2α-epoxy-4β-hydroxy-5αH,7αH,8αH,10αH-guaia-11(13)-en-8β,12-olide (**2**) and 5,10-*epi*-2,3-dihydroaromatin (**3**). The structures were assigned on the basis of extensive 1 and 2D NMR experiments. Compound **3** exhibited weak antimycobacterial activity against *Mycobacterium phlei* with a minimum inhibitory concentration of 0.52 mM and cytotoxicity (IC_{50} of 5.8 ± 0.2 μM) in a human bladder carcinoma cell line, EJ-138.

1. Introduction

Pulicaria crispa (Forssk.) Oliv. [syn. *Pulicaria undulata* (L.) C.A.Mey., *Francoeuria crispa* (Forssk.) Cass.] is an annual herb or sometimes a perennial sub shrub, belonging to the family Asteraceae, producing small bright yellow flowers. This species is distributed in Saudi Arabia, Kuwait, Iran, Iraq, Egypt, Afghanistan, Pakistan, India and parts of north and west tropical Africa (Boulos, 2002; Al-Rawi, 1987). *P. crispa* is a medicinal plant used by people of southern Egypt and Saudi Arabia to treat inflammation and also as an insect repellent (Ross et al., 1997) and is also used as an herbal tea. Phytochemical studies of this herb have identified it to be a rich source of sesquiterpene lactones of the guaianolide (Dendougui et al., 2000), eudesmanolide (San Feliciano et al., 1989) and xanthanolide classes as well as kaurane diterpenes (Abdel-Mogib et al., 1990).

2. Results and discussion

Compound **1** (Fig. 1) was isolated as a colourless oil and a molecular formula of $C_{15}H_{20}O_4$ $[M]^+$ (264.1355) was assigned by HR-EIMS. The ^1H and ^{13}C NMR signals (Table 1) were characteristic of a guaianolide sesquiterpene, which is distinctive of the genus *Pulicaria* (Dendougui et al., 2000). Assuming a guaianolide skeleton for compound **1**, a methyl singlet attributed to C-15 exhibited a ^2J correlation to C-4 and ^3J correlations to a methylene carbon (C-3) and an olefinic quaternary carbon (C-5). By inspection of the COSY spectrum, the methylene hydrogens at C-3 coupled to a deshielded oxymethine hydrogen (H-2, δ_H 4.86), which in turn gave a ^2J correlation to a second olefinic quaternary carbon (δ_C 140.8, C-1) as well as a ^3J correlation to C-5. H_2-3 confirmed the assignment of a cyclopentene ring system with two ^3J correlations to C-1 and C-5. The downfield appearance of C-2 and C-4 in the ^{13}C NMR spectrum as well as H-2, in the ^1H NMR spectrum indicated that hydroxyl groups should be placed at these positions therefore completing ring A. The oxymethine group at position 2 was allylic causing the appearance of both the carbon and hydrogen signals to be shifted further downfield. A second methyl (C-14) appearing as a doublet in the ^1H spectrum gave a COSY coupling to a methine proton (H-10) and in the HMBC spectrum exhibited two ^3J correlations to C-1 and C-9, placing the methyl adjacent to ring A. H_2-9 gave a strong COSY coupling towards a downfield methine hydrogen (H-8, δ_H 4.76) which in turn coupled to H-7. The 7-membered ring of ring B was completed by a COSY coupling between H-7 and methylene H_2-6 and by ^2J and ^3J HMBC correlations from H_2-6 and H-7 to C-5, respectively. A ^3J correlation between H-7 and an *exo*-cyclic olefinic methylene carbon (C-13) placed this *exo*-cyclic methylene on ring C of the molecule. ^2J and ^3J HMBC signals between the hydrogens of this *exo*-methylene and its olefinic quaternary partner (C-11) and an ester carbonyl carbon (C-12) confirmed the position of these groups as part of the guaianolide ring-C lactone. The deshielded nature of H-8, indicated that it was attached to a carbon bearing an oxygen, completing ring C by forming a 5-membered lactone ring system between C-8 and C-12.

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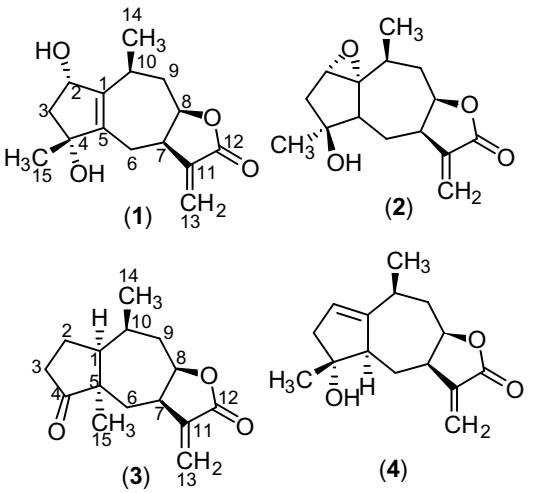

Fig. 1. Structures of **1–4**.

The relative stereochemistry of **1** was determined by inspection of the ^{1}H and NOESY spectra. An NOE correlation between H-7 and H-8 indicated that these two hydrogens are on the same face of the molecule. As only the coupling constants could be measured for H-8 and not for H-7, a model of this molecule indicated that these hydrogens were *cis* (α-oriented). This correlated well with one of the coupling constants measured for H-8 of 8.5 Hz and helped to confirm the *cis* orientation. A similar coupling constant measured for the known compound **4** also corroborated this assignment. A 1,3 NOE interaction between H-8 and H-10 allowed the assignment of H-10 as being α, therefore methyl-14 must be positioned in a β-orientation. A third NOE between methyl-14 and H-2 placed H-2 in a β-orientation and so the hydroxyl attached to C-2 must be α. An NOE between H-2 and the downfield hydrogen of the methylene at position 3 (δ_H 2.35) meant that this proton must be on the same face of the molecule (β). Thus a 1,3 interaction between H_β-3 and methyl-15 showed that this group should also be assigned as β. This was further confirmed by an NOE correlation between methyl-15 and both hydrogens of H_2-6. Compound **1** is therefore assigned as 2α,4α-dihydroxy-7αH,8αH,10αH-guaia-1(5),11(13)-dien-8β,12-olide and is reported here for the first time.

Compound **2** was isolated as a pale yellow oil and the HR-EIMS revealed a molecular formula of $C_{15}H_{20}O_4$ $[M]^+$ (264.1363). The NMR data were similar to those of **1**. The ^{13}C NMR spectrum indicated the presence of 15 carbons, including a carbonyl, two olefinic and two oxygenated quaternary carbons. The ^{1}H NMR spectrum provided signals for an *exo*-methylene group, two methyls and two deshielded methine hydrogens. The ^{1}H spectrum provided evidence to suggest that **2** was a guaianolide due to the similarity in the spectral data when compared with compound **1**. The structure of **2** (Fig. 1) was closely related to that of **1**, except for the absence of a double bond between C-1 and C-5 and the absence of an hydroxyl group attached at C-2. Instead ring A of **2** was saturated with the formation of an epoxide between C-1 and C-2. The remainder of the guaianolide structure was identical to that of **1** with similar HMBC and COSY correlations. The ^{13}C resonances at δ_C 72.4, (C-1) and δ_C 59.6, (C-2) were characteristic for epoxide carbons (Trifunovic et al., 2006) especially for methine C-2. The hydroxyl hydrogen was also detected in the ^{1}H spectrum as a sharp singlet and provided information on point of attachment, with a ^2J correlation to C-4 and a ^3J correlation to C-15. This further corroborated the structure of ring-A as a cyclopentane ring. The ^{1}H NMR data for **2** were in close agreement with that of the literature (Zdero et al., 1988). However, the NOESY spectrum provided evidence to indicate that **2** was in fact the epimer of the guaianolide isolated by Zdero et al., at the C-4 position and stereochemistry at C-2, 7, 8 and 10 were identical to that in **1**.

H-7 showed an NOE to H-5, which in turn gave a 1,3 interaction with the methyl hydrogens of C-15. This indicated that both H-5 and H_3-15 must also be on the same face of the molecule as H-7 in an α-orientation. The methylene hydrogens at C-6 each gave an NOE to H_3-15 and a molecular model of this molecule showed that these hydrogens were equidistant from the methyl group further confirming the relative stereochemical assignment at C-4. The hydroxyl group must therefore be in a β-orientation. This is the point of difference between the relative stereochemistry of **2** and that assigned by Zdero et al. The guaianolide detailed in the literature was described with the methyl group in a β-orientation and the hydroxyl group in an α-orientation. The C-14 methyl hydrogens gave a 1,3 interaction to the epoxide methine (H-2) placing this

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) spectral data of **1–4**. **1** and **4** were recorded in CD_3OD, **2** was recorded in $CDCl_3$ and **3** was recorded in C_6D_6

Position	1		2		3		4	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	–	140.8	–	72.4	1.16 *m*	48.6	–	151.1
2	4.86 *m*	87.4	3.59 *s*	59.6	0.97 *ddd* (9.0, 3.0) 1.42 *dddd* (12.5, 9.5, 6.5, 1.0)	24.6	5.37 t (1.5)	120.3
3	2.09 *dd* (13.5, 4.5) 2.35 *dd* (13.5, 7.0)	46.0	1.77 *d* (14.5) 2.06 *d* (15.0)	40.6	1.72 *ddd* 2.01 *m*	35.6	2.37 *dd* (16.0)	47.5
4	–	81.6	–	78.2	–	222.4	–	82.1
5	–	144.9	2.02 *dd* (13.0, 2.0)	54.9	–	50.2	2.48 *d* (12.5)	59.7
6	2.41 *dd* (15.0, 5.0) 2.46 *ddt* (15.0, 5.0, 2.5)	25.5	1.38 *bdd* (12.5) 1.79 *m*	30.6	1.11 *dd* (13.0, 2.5) 2.19 *m*	35.4	1.46 *dd* (12.5) 1.99 *m*	32.7
7	3.44 *m*	44.1	3.21 *m*	41.9	2.21 *m*	45.1	3.37 *ddd* (12.0, 9.5, 4.0)	43.3
8	4.76 *ddd* (12.5, 8.5, 4.5)	81.5	4.72 *ddd* (12.0, 7.5, 4.5)	79.1	3.69 *ddd* (12.0, 9.0, 3.0)	81.0	4.82 *m*	82.6
9	1.99 *m*	37.2	1.73 *d* (14.0) 2.07 *dd* (13.5, 0.5)	35.3	0.95 *d* (12.5) 2.02 *d* (17.5)	44.8	1.56 *dd* (12.0) 1.93 *m*	39.1
10	2.65 *dq* (12.0, 7.0, 2.0)	31.0	2.14 *m*	30.0	1.24 *m*	30.0	2.24 *bs*	32.4
11	–	141.6	–	140.1	–	142.1	–	142.4
12	–	172.0	–	169.5	–	169.9	–	172.1
13	5.75 *d* (2.5) 6.21 *d* (3.0)	123.1	5.65 *d* (2.0) 6.29 *d* (2.5)	123.3	4.99 *d* (3.0) 6.04 *d* (3.5)	119.3	5.73 *d* (2.5) 6.20 *d* (3.0)	122.9
14	1.18 *d* (7.0)	20.6	0.90 *d* (7.0)	17.2	0.64 *d* (6.5)	20.4	1.22 *d* (6.5)	20.6
15	1.21 *s*	27.0	1.17 *s*	23.5	0.56 *s*	22.4	1.25 *s*	24.7
4-OH			3.70 *s*					

proton on the same face of the molecule as H_3-14 (β). Therefore the epoxide must be α-oriented and this follows the representation of the epimeric form of the molecule described by Zdero et al. (1988). Compound **2** is assigned as 1α,2α-epoxy-4β-hydroxy-5αH,7αH,8αϖ H,10αH-guaia-11(13)-en-8β,12-olide and is reported here for the first time.

Compound **3** was isolated as a white amorphous powder and the accurate EI-MS in the positive mode provided the molecular ion of *m/z* 248.1412 to establish a molecular formula of $C_{15}H_{20}O_3$. The ^{1}H NMR and ^{13}C NMR spectra provided signals for a sesquiterpene, but this time for a pseudoguaianolide sesquiterpene. The HMBC spectrum provided similar signals as for the previous guaianolide sesquiterpenes discussed above, again with notable differences occurring in ring A. The position of methyl-15 strongly indicated that a Wagner–Meerwein rearrangement had taken place, moving this group from C-4 to C-5 of the molecule to give a pseudoguaianolide. The normal biosynthetic pathway from the C_{15} precursor farnesyl pyrophosphate would place this methyl group at C-4. However, in the case of **3**, C-4 had been oxidised to a ketonic carbonyl. Methyl-15 appeared as a singlet and gave a ^2J correlation to a quaternary carbon C-5 (δ_C 50.2) as well as ^3J correlations to C-1 (δ_C 48.6), C-6 (δ_C 35.4) and the carbonyl of C-4 (δ_C 222.4). This ketonic carbonyl was highly deshielded and this was due to the highly strained nature of the cyclopentane ring (ring A). The methylene hydrogens of C-3 gave a COSY correlation with H_2-2, which in turn gave a further COSY correlation to H-1. The methine proton, H-1, also gave a COSY correlation to H-10, which in turn gave a COSY signal to H_2-9 and to a methyl doublet (H_3-14) placing these groups here. The methylene protons attached at C-9 then gave a COSY correlation to the oxymethine proton, H-8. A COSY correlation between H-8 and H-7 indicated that they should be placed at the ring junction of ring B and C as seen in compounds **1** and **2**. This was confirmed by an allylic coupling detected between H-7 and the *exo*-methylene hydrogens H_2-13. The COSY correlations detected for **3** were similar to those of the previously discussed guaianolides.

The relative stereochemistry of **3** (Fig. 1) was achieved by NOE's detected in the NOESY spectrum along with analysis of measured coupling constants. An NOE between H-8 and H-7 placed these protons on the same face of the molecule in an α-orientation. The large coupling constant (9.0 Hz) again indicated that these hydrogens were *cis*. A second NOE between H-8 and H-10 also placed these hydrogens on the same face of the molecule (α), therefore methyl-14 must be β-oriented. This was further confirmed by NOE's between both H_2-2α and H_2-2β to methyl-14. A molecular model showed that the methylene hydrogens were equidistant with respect to methyl-14, whereas if the methyl group were α-orientated this would not be possible. An NOE between H-8 and H-1 also placed this hydrogen in an α-orientation, thus confirming the relative stereochemistry at this position. Finally, methyl-15 exhibited NOE's with H-10 and H_2-3α placing this group on the same face of the molecule as H-1, H-7, H-8 and H-10 (α).

Compound **3** has been isolated previously (Abdel-Mogib et al., 1990; Bohlmann and Mahanta, 1979; Rustaiyan et al., 1987), however the relative stereochemistry of this compound differs from all of these compounds referred to in at least one position. All published literature has quoted the ^{1}H NMR data in deuterated chloroform, which suffers from signal overlap. However, for the purpose of comparing the ^{1}H NMR data with that of the literature, **3** was also analysed in deuterated chloroform. The ^{1}H NMR data for **3** was in agreement with that of the literature for 2,3-dihydroaromatin (Merfort and Wendisch, 1993) with the exception of H-7 and H-8 which were reported to be further downfield. The downfield values reported for these hydrogens are consistent with previous literature articles for guaianolides that have these protons in a *cis* (α) arrangement (Zdero et al., 1988), whereas they appear more upfield when H-7 (~2.75 ppm) and H-8 (~4.30 ppm) are *trans* (Abdel-Mogib et al., 1990; Rustaiyan et al., 1987). To confirm this relative assignment the NOESY experiment for **3** acquired in deuterated benzene again indicated that H-7 and H-8 should be *cis* (α-oriented). Compound **3** differs from 2,3-dihydroaromatin at positions 5 and 10. The methyl groups attached to these carbons in compound **3** are β- and α-oriented, respectively. However in 2,3-dihydroaromatin the methyl groups at positions 5 and 10 are α- and β-oriented, respectively. The pseudoguaianolide **3** is reported here for the first time as 5,10-*epi*-2,3-dihydroaromatin. 5,10-*epi*-2,3-Dihydroaromatin also exhibited weak antimycobacterial activity against *Mycobacterium phlei* with a minimum inhibitory concentration (MIC) of 0.52 mM. Whilst the activity recorded is weak it can provide a starting point for analogues with greater activity, particularly as the known guaianolide aromaticin, similar to **3**, has been reported to exert an antimycobacterial effect with an MIC of 0.064 mM (Cantrell et al., 2001; Copp, 2003).

Compound **3** was evaluated in vitro for anti-cancer activity in an established human bladder carcinoma cell line, EJ-138 and demonstrated promising activity in this cell line, with an IC_{50} of 5.8 ± 0.2 μM.

Compound **4** was isolated as a colourless oil and solved for a molecular formula of $C_{15}H_{20}O_3$ by ESI-MS. The ^{1}H NMR spectral data were identical to those of 1,2-dehydro-1,10α-dihydropseudoivalin (Zdero et al., 1988) which has previously been isolated from *Pulicaria sicula* and this is the first report of the ^{13}C NMR data for this compound and the first report of the full NMR data in deuterated methanol (Table 1).

3. Experimental

3.1. General experimental techniques

NMR spectra were recorded on a Bruker AVANCE 500 MHz spectrometer. Chemical shift values (δ) were reported in parts per million (ppm) relative to appropriate internal solvent standard and coupling constants (J values) are given in Hertz. Mass spectra were recorded on a Finnigan MAT 95 high resolution, double focusing, magnetic sector mass spectrometer. Accurate mass measurement was achieved using voltage scanning of the accelerating voltage. This was nominally 5 kV and an internal reference of heptacosa was used. Resolution was set between 5000 and 10,000.

IR spectra were recorded on a Nicolet 360 FT-IR spectrophotometer and UV spectra on a Thermo Electron Corporation Helios spectrophotometer.

The cancer cell growth inhibition assay details are provided in the supporting information.

3.2. Plant material

The plant material used for this study was collected from KSIR field station, in the Kebd area of Kuwait on the 27th of April 1999. The material was identified by K.T. Mathew and a voucher specimen (KTM 4612, collected by Simon Gibbons and K.T. Mathew) is deposited at the Kuwait University Herbarium (KTUH).

3.3. Extraction and isolation

The air dried aerial parts of *P. crispa* (185 g) were coarsely powdered and sequentially extracted in a Soxhlet apparatus with hexane (3.5 L), chloroform (3.5 L) and finally methanol (3.5 L). The hexane extract (6.7 g) was subjected to vacuum liquid chromatography (VLC) on silica gel (10 g) eluting with hexane containing 10% increments of ethyl acetate to yield 12 fractions. The fraction eluted with 80% ethyl acetate underwent further fractionation by Sephadex LH-20 chromatography eluting with dichloromethane (DCM) followed

by methanol. Final purification of the methanol fraction by multiple preparative thin layer chromatography (TLC) in reverse phase mode (C_{18}; H_2O:methanol, 1:1) (two times) afforded **1** (7.6 mg). Compound **4** was purified from the same VLC fraction as **1**. DCM fraction 3 of Sephadex LH-20 chromatography was subjected to solid phase extraction using a 6:4 methanol:H_2O system to purify **4** (12 mg). VLC fraction 7 (6:4 ethyl acetate:hexane) was subjected to Sephadex LH-20 chromatography eluting with DCM followed by multiple preparative TLC of fraction 2 in normal phase mode (65:35 hexane:ethyl acetate; two times). This afforded compound **2** (9.5 mg). VLC fraction 6 (1:1 hexane:ethyl acetate) was also subjected to Sephadex LH-20 chromatography, eluting with DCM. On addition of hexane to fraction 2 a white precipitate was observed. Three washes of the precipitate with hexane followed by decanting the supernatant enabled the purification of compound **3** (100 mg).

3.4. 2α,4α-Dihydroxy-7αH,8αH,10αH-guaia-1(5),11(13)-dien-8β,12-olide (**1**)

Colourless oil; $[\alpha]_D^{23} + 37.0^\circ$ (*c* 0.378, $CHCl_3$); UV (CH_3OH): λ_{max}: ($\log\varepsilon$) 218 (8.56) nm; IR ν_{max} (thin film) cm^{-1}: 3352, 2963, 1748, 1653, 1276, 986; ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) see Table 1. HREIMS *m/z* 264.1355 (calc. for $C_{15}H_{20}O_4$, 264.1362).

3.5. 1α,2α-Epoxy-4β-hydroxy-5αH,7αH,8αH,10αH-guaia-11(13)-en-8β,12-olide (**2**)

Pale yellow oil; $[\alpha]_D^{23} + 33.7^\circ$ (*c* 0.475, $CHCl_3$); UV (CH_3OH): λ_{max}: ($\log\varepsilon$) 214 (8.52) nm; IR ν_{max} (thin film) cm^{-1}: 3566, 2933, 1761, 1653, 1271, 1128; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) see Table 1. HREIMS *m/z* 264.1363 (calc. for $C_{15}H_{20}O_4$, 264.1362).

3.6. 5,10-epi-2,3-Dihydroaromatin (**3**)

White amorphous powder; $[\alpha]_D^{25} + 104.8^\circ$ (*c* 4.75, $CHCl_3$); UV (ACN): λ_{max}: ($\log\varepsilon$) 222 (7.20) nm; IR ν_{max} (thin film) cm^{-1}: 2968, 2931, 1763, 1736, 1125, 996; ^1H NMR (500 MHz, C_6D_6) and ^{13}C NMR (125 MHz, C_6D_6) see Table 1. HREIMS *m/z* 248.1412 (calc. for $C_{15}H_{20}O_3$, 248.1413).

3.7. 1,2-Dehydro-1,10α-dihydropseudoivalin (**4**)

Colourless oil; $[\alpha]_D^{24} + 23.9^\circ$ (*c* 0.418, $CHCl_3$); UV (CH_3OH): λ_{max}: 218 nm; IR ν_{max} (thin film) cm^{-1}: 3420, 2963, 1759, 1270, 1128, 969; ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) see Table 1. Negative ESI-MS: *m/z* = 293.1 $[M-H+2Na]^-$.

3.8. Bacterial strain and antibacterial assay

M. phlei ATCC 11758 was obtained from NTCC. *M. phlei* was cultured on Columbia blood agar (Oxoid) supplemented with 7% defibrinated horse blood (Oxoid) and incubated for 72 h prior to MIC determination. A bacterial inoculum equivalent to 5×10^5 cfu/mL was prepared in normal saline using the 0.5 McFarland turbidity standard followed by dilution. The MIC was recorded as the lowest concentration at which no bacterial growth was observed (Gibbons and Udo, 2000). Ethambutol was used as a positive control, whilst growth and sterile controls were also performed.

3.9. Cancer cell line

The EJ-138 human bladder carcinoma cell line (ECACC, Salisbury, UK) was cultured in RPMI 1640 cell culture medium supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine and 10% fetal bovine serum (all from Sigma, Poole, UK).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.03.012.

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