Enzyme Inhibiting Terpenoids from *Amberboa ramosa*

Sher B. Khan[a], Abdul Malik[a], Nighat Afza[b], Nusrat Jahan[a], Azhar U. Haq[a], Zaheer Ahmed[a], Sarfraz A. Nawaz[a], and Mohammed Iqbal Choudhary[a]

[a] International Centre for Chemical Sciences, H. E. J. Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan
[b] Pharmaceutical Research Centre, PCSIR Labs Complex Karachi, Karachi-75280, Pakistan

Reprint requests to Prof. Abdul Malik. Fax: (+92)-21-9243190-91.
E-mail: hej@digicom.net.pk and hejric@digicom.net.pk

Z. Naturforsch. **59b,** 579 – 583 (2004); received October 20, 2003

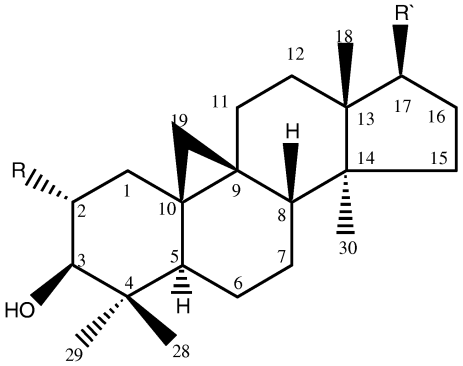
Two new cycloartane type triterpenes **1** and **2** have been isolated from *Amberboa ramosa*. Their structures were established as (22*R*)-cycloart-20, 25-dien-2α,3β,22α-triol **1** and (22*R*)-cycloart-23-ene-3β,22α,25-triol **2** through spectroscopic studies including 2D-NMR. The known compounds 3,4-epoxyguaia-1(10), 11(13)-dien-6, 12-olide **3**, and 5-hydroxy 7,8,2',3'-tetramethoxyflavone **4** are also reported for the first time from this species. The compounds **1** and **2** displayed inhibitory potential against butyrylcholinesterase.

Key words: Amberboa ramose, Compositae, Cycloartanes, Enzyme Inhibition

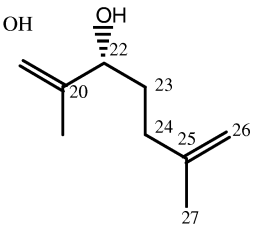
Introduction

The genus *Amberboa* belongs to the family Compositae and comprises six species. One of them is *Amberboa ramosa* Jafri which is an annual herbaceous plant found in India and Pakistan. The plant has tonic, aperient, febrifuge, deobstruent, cytotoxic and antibacterial activities [1]. Previously, triterpenes have been reported from *Amberboa ramosa* [2]. Herein we report the isolation and structure elucidation of two terpenoids (22*R*)-cycloart-20, 25-dien-2α,3β,22α-triol **1** and (22*R*)-cycloart-23-ene-3β,22α,25-triol **2** along with known compounds 3,4-epoxyguaia-1(10), 11(13)-dien-6,12-olide **3** [3] and 5-hydroxy 7,8,2',3'-tetramethoxyflavone [4, 5], respectively.

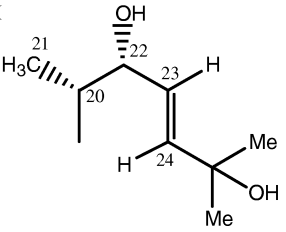
Butyrylcholinesterase (BChE, EC 3.1.1.8) inhibition may be an effective method for the treatment of *Alzheimer's* disease (AD) and related dementias [6]. These inhibitors may act as potential leads in the discovery of clinically useful inhibitors for nervous system disorders, particularly by reducing memory deficiency in *Alzheimer's* disease patients by potentiating and affecting the cholinergic transmission process. The inhibitory activity of **1** and **2** has been determined by the method described in the experimental and the results are summarized in Table 2.



1 R = OH
R` =



2 R = H
R` =



Result and Discussion

Compounds **1** and **2** were isolated from the chloroform soluble fraction of the methanolic extract of the ground and shade dried plant material. Compound 1 was isolated as colourless crystals. The molecular ion peak at *m/z* 456.3547 in the HRMS indicated its molecular formula to be $C_{30}H_{48}O_3$ (calcd. 456.3605). Absorption bands at (3600 – 3450 cm^{-1}), 3045 cm^{-1}, 1650 and 890 cm^{-1} in the IR spectrum of **1** suggested the presence of a hydroxyl, a cyclopropane ring and a terminal methylene group respectively. The ^{1}H NMR spectrum displayed signals corresponding to five tertiary methyl groups at δ_H 0.92, 0.94, 0.95($CH_3 \times 2$), and 1.70. A pair of doublets at δ_H 0.30 and 0.52 ($J =$ 4.2 Hz) was indicative of a cyclopropane ring bearing two non-equivalent hydrogen atoms. Broad singlets at δ_H 4.50, 4.60, 4.80 and 4.90 (1H, each) were assigned to the terminal methylene protons. The doublet of double doublet at δ_H 3.98, doublet at δ_H 3.20 and a triplet at δ_H 4.01 were due to the protons attached to the carbons bearing hydroxyl groups. The BB ^{13}C NMR and DEPT experiments revealed the presence of five methyl, twelve methylene, six methine and seven quaternary carbons. It included the olefinic carbons at δc 152.5, 147.8, 111.4 and 110.8 and the oxygen bearing carbons at δc 80.6, 76.8 and 68.0, respectively. Further information could be obtained from the mass spectrum which showed daughter ion at *m/z* 369.3011($C_{25}H_{37}O_2$) corresponding to the elimination of C_5H_9 from $(M\text{-}18]^+$ peak, which is characteristic of 4,4-dimethyl-9,19-cyclosterol [7]. Another characteristic fragmentation involved elimination of the ring A [7, 8] producing an ion at *m/z* 300.3211 ($C_{21}H_{32}O$) and another peak at *m/z* 175.1523 ($C_{13}H_{19}$), the latter arising from the loss of the $C_8H_{15}O$ side chain from the fragment at *m/z* 300.3211. The fragmentation pattern was characteristic of cycloartane type triterpene with one hydroxyl function in the side chain and the other two hydroxyl groups in ring A. In the ^{1}H-^{1}H homonuclear chemical shift correlation spectrum (COSY-45°) the comparatively upfield proton at δ_H 3.20 showed connectivity with only one proton at δ_H 3.98. It could be assigned to C-3 on biogenetic grounds. The large coupling ($J_{ax,ax} = 11.8$ Hz) allowed us to assign β and equatorial orientation to the hydroxyl group at C-3. The proton at δ_H 3.98 showed connectivity to H-3 at δ_H 3.20 and also with two further protons. It could be assigned to hydroxyl bearing C-2 in β and axial orientation, based on larger coupling constants (11.8, 8.4 and 4.3 Hz). The possible location of the hydroxyl group in the side chain were either at C-22 or C-24, the latter could be eliminated by HMBC experiments where the signal at δ_H 4.01 showed ^2J coupling with C-20 at δc 152.5 and ^3J coupling with C-21 at δc 110.8, respectively. The presence of a double bond at C-25 was indicated by the absence of the characteristic 6H doublet of an isopropyl group and the presence of vinyl methyl singlet at δ_H 1.70. HMBC spectra (Fig. 1) and spin decoupling experiments, combined with nOe difference spectroscopy allowed complete assignment of the stereochemistry of **1**. The cyclopropane protons showed nOe's with signals at δ_H 0.95 (H_3-18) and 0.92 (H_3-29) (irradiation of H-19β at δ 0.30) and to H-2β and H_3-18 (irradiation of H-19α at 0.52). The H_3-18 showed nOe's with both H-19β and H-19α as well as H-22β. The relative configuration at C-22 was concluded to be *R* by comparing the C_{13}-chemical shift of δc 76.8 for C-22 which appeared more upfield than the corresponding *S* epimer [9, 10]. Thus, the structure of **1** was concluded to be (22*R*)-cycloart-20, 25-dien-2α,3β,22α triol.

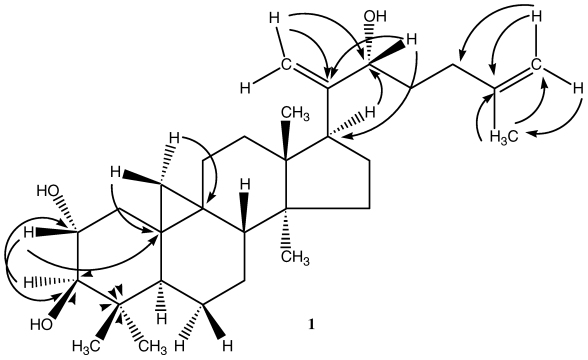


Fig. 1. Important HMBC interactions of **1**.

Compound **2** was isolated as colourless crystals, showed a molecular ion peak at *m/z* 458.3842 in the HRMS corresponding to $C_{30}H_{50}O_3$ (calcd. 458.3762). Its IR spectrum revealed the presence of hydroxyl groups (3590, 3440 cm^{-1}), cyclopropyl ring (3040 cm^{-1}) and a disubstituted double bond (1650 – 1645, 950 cm^{-1}). The ^{1}H NMR spectrum showed signals due to six tertiary methyl groups at δ_H 0.80, 0.85, 0.95 ($CH_3 \times 2$) and δ_H 1.29 ($CH_3 \times 2$) along with one secondary methyl group at δ_H 0.84 (d, $J = 6.4$ Hz). The presence of cyclopropane ring was confirmed by the presence of characteristic double doublet at δ_H 0.31 and 0.52 ($J = 4.2$ Hz) for two non-equivalent protons at C-19, a double doublet at δ_H 3.20 ($J = 9.9$ and 4.5 Hz)

were due to the proton geminal to a hydroxyl group. In ^{1}H-^{1}H correlated spectroscopy it showed the large J value (9.9, 4.5 Hz) indicating the β and equatorial orientation of OH. The doublet at δ_H 4.06 ($J = 5.6$) indicated another proton attached to the carbon bearing hydroxyl group. A one proton double doublet at δ_H 6.50 ($J = 15.6$, 5.6 Hz), and another one proton doublet at δ_H 6.58 ($J = 15.6$) could be assigned to double bond in *E* configuration. ^{13}C NMR and DEPT experiments revealed the presence of 7 methyl, 9 methylene, 8 methine and six quaternary carbons. The mass fragmentation pattern was very similar to that of **1** showing characteristic peaks at *m/z* 440 $[M\text{-}H_2O]^+$, 433 $[M\text{-}CH_3]^+$, 422 $[M\text{-}2H_2O]^+$. Further ions were observed at *m/z* 318 $[C_{23}H_{34}O_2]^+$, generated due to loss of ring A, 300 $[C_{21}H_{34}O_2\text{-}H_2O]^+$, while the peak at *m/z* 175 $[C_{21}H_{34}O_2\text{-}C_8H_{15}O_2]^+$ resulted by the loss of the side chain from C-17, revealing the presence of two OH groups and a double bond in the side chain. The doublet at δ_H 0.84 was assigned to H_3-21 showing a cross peak with H-20 at δ_H 1.45 in COSY-45° spectrum. The downfield proton at δ_H 4.06 showed correlation with H-20 at δ_H 1.45 and the olefinic proton at δ_H 6.50 allowing us to assign the hydroxyl group and the double bond to C-22 and C-23, respectively. The remaining hydroxyl group was assigned to C-25 as the methyl groups attached to it shifted downfield to δc 29.8 and 29.7, respectively.

The configurations of hydrogens attached to carbons C-20 and C-22 could be confirmed as β by nOe difference spectroscopy in which H_3-18β showed strong nOe with both β protons at C-20 and C-22, respectively. Thus compound **2** could be assigned the structure (22*R*)-cycloart-23-ene-3β, 22α, 25-triol. The HMBC correlations (Fig. 2) fully supported the assigned structure.

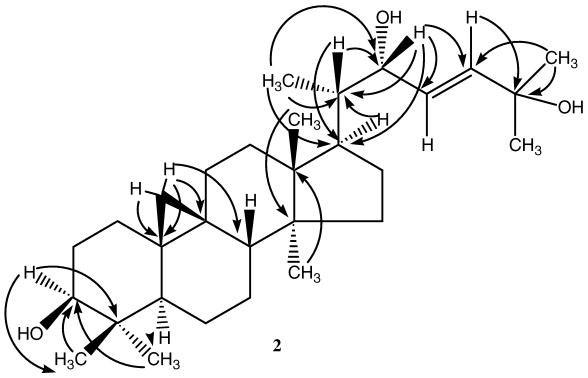


Fig. 2. Important HMBC interactions of **2**.

Experimental Section

General experimental procedures. – Optical rotations were measured on a JASCO DIP-360 polarimeter. IR spectra were recorded on a 460 Shimadzu spectrometer. EIMS and HRFABMS were recorded on JMS-HX-110 with a data system and on JMS-DA 500 mass spectrometers. The ^{1}H and ^{13}C NMR, HMQC, and HMBC spectra were recorded on Bruker spectrometers operating at 400 MHz for ^{1}H and 100.6 MHz for ^{13}C NMR, respectively. The chemical shift values are reported in ppm (δ) units and the coupling constants (J) are in Hz. Aluminum sheets precoated with silica gel 60 F_{254} (20 × 20 cm, 0.2 mm thick; E-Merck) were used for TLC and flash silica (230 – 400 mesh) was used for column chromatography.

Plant material. – *Amberbao ramosa* (Compositae), whole plant was collected in June 2002, from Karachi (Pakistan) and identified by Dr. Surraiya Khatoon, Plant Taxonomist, Department of Botany, University of Karachi, where a voucher specimen has been deposited.

The dried plant material (20 kg) was extracted thrice with methanol at room temperature. The methanol extract was evaporated under reduced pressure to afford a dark residue which was suspended in water and successively extracted with *n*-hexane, chloroform, ethylacetate and *n*-butanol. The chloroform fraction (150 g) was subjected to column chromatography over silica gel, successively eluting with *n*-hexane, *n*-hexane-ethylacetate, ethylacetate, ethylacetate-methanol in increasing order of polarity. The fraction which eluted with *n*-hexane-ethylacetate (85:15) showed two major and two minor spots on TLC. It was further subjected to CC (flash silica, 230 – 400 mesh) using *n*-hexane-ethylacetate (87:13) as eluent to afford compound **1** (12 mg) and compound **2** (15 mg). The fraction which eluted with *n*-hexane-ethylacetate (80:20) showed two major spots on TLC. It was further subjected to CC (flash silica, 230 – 400 mesh) using *n*-hexane-ethylacetate (85:15) as eluent to afford compound **3** (15 mg) and *n*-hexane-ethylacetate (80:20) to provide **4** (18 mg).

Compound **1**. – Colourless crystalline. – M. p. 186 – 188 °C. – $[\alpha]_D^{30} + 36.4°$ ($c = 0.022$, $CDCl_3$). – IR (KBr) $\tilde{\nu} = 3600 - 3450$, 3045, 1650, 890 cm^{-1}. – ^{1}H NMR and ^{13}C NMR, see Table 1. – MS (EI, 70 eV): *m/z* (%) = 456 (24) (M^+), 438 (35) $[M^+\text{-}H_2O]$, 423 (25) $[M^+\text{-}H_2O\text{-Me}]$, 420 (10) $(M^+\text{-}2H_2O)$, 387 (8) $[M^+\text{-}C_5H_9]$, 369 (14) $[M^+\text{-}C_5H_9\text{-}H_2O]$, 313 (90) [$M^+$-18-side chain], 282 $[C_{21}H_{32}O^+\text{-}H_2O]$, 175 (50) $[C_{21}H_{32}O^+\text{-}C_8H_{13}O]$. – HREIMS: $m/z = 456.3547$ (calcd. 456.3605 for $C_{30}H_{48}O_3$).

Compound **2**. – Colourless crystalline. – M. p. 194 – 196 °C. – $[\alpha]_D^{30} + 43.4°$ ($c = 0.023$, $CDCl_3$). – IR (KBr) $\tilde{\nu} =$ 3590, 3440, 3040, 1650 – 1645, 950 cm^{-1}. – ^{1}H NMR and ^{13}C NMR, see Table 1. – MS (EI, 70 eV): *m/z* (%) = 458 (12) (M^+), 443 (10) [M^+-Me], 440 $[M^+\text{-}H_2O]$, 425 (18) [M^+-

C	**1** (δC)	**1** (δ_H)	**1** HMBC (H→C)	**2** (δC)	**2** (δ_H)	**2** HMBC (H→C)
1	40.1 (CH_2)			31.9 (CH_2)		
2	68.0 (CH)	3.98 ddd (11.8, 8.6,4.3)	C-3, C-4, C-10,	30.4 (CH_2)		
3	80.6 (CH)	3.20 d (11.8)	C-2, C-4	78.8 (CH)	3.20 dd (9.9,4.5)	C-4, C-28, C-29
4	37.4 (C)			40.5 (C)		
5	47.1 (CH)			47.1 (CH)		
6	21.1 (CH_2)			21.1 (CH_2)		
7	28.1 (CH_2)			28.0 (CH_2)		
8	47.9 (CH)			47.9 (CH)		
9	20.0 (C)			20.0 (C)		
10	26.2 (C)			26.1 (C)		
11	26.0 (CH_2)			26.4 (CH_2)		
12	35.6 (CH_2)			35.6 (CH_2)		
13	45.3 (C)			45.1 (C)		
14	48.8 (C)			48.8 (C)		
15	32.9 (CH_2)			32.0 (CH_2)		
16	26.5 (CH_2)			25.9 (CH_2)		
17	52.2 (CH)			51.9 (CH)		
18	18.0 (CH_3)	0.95 s	C-12, C14, C-17	18.1 (CH_3)	0.95 s	C-14, C-17,
19	30.1 (C)	0.30 d (4.2) 0.52 d (4.2)	C-9, C-10	29.8 (C)	0.52 d (4.2),0.31 d(4.2)	C-8, C-9, C-10,
20	152.5 (C)			36.0 (CH)		
21	110.8 (CH_2)	4.50 s 4.60 s	C-20, C-22	18.3 (CH_3)	0.84 d (6.4)	C-17, C-20, C-22
22	76.8 (CH)	4.01 t (6.5)	C-17, C-20	76.8 (CH)	4.06 d (5.6)	C-17, C-20 C-23, C-24
23	32.0 (CH_2)			125.8 (CH)	6.50 dd (15.6,5.6)	C-22, C-24
24	34.1 (CH_2)			140.2 (CH)	6.58 d (15.6)	C-25
25	147.8 (C)			70.0 (C)		
26	111.4 (CH_2)	4.80 s 4.90 s	C-24, C-25, C-27	29.8 (CH_3)	1.29 s	C-24, C-25
27	17.2 (CH_3)	1.70 s	C-25, C-26	29.7 (CH_3)	1.29 s	C-24, C-25
28	19.3 (CH_3)	0.94 s	C-3, C-4	25.4 (CH_3)	0.85 s	C-3, C-4
29	25.4 (CH_3)	0.92 s	C-3, C-4	19.3 (CH_3)	0.80 s	C-3, C-4
30	14.0 (CH_3)	0.95 s	C-13, C-14, C-15	14.0 (CH_3)	0.95 s	

Table 1. ^{1}H- and ^{13}C NMR spectral data and HMBC correlations of Compounds **1** and **2**.

[a] ^{13}C NMR carried out at 100 MHz; [b] ^{1}H NMR carried out at 400 MHz; [c] HMBC carried out at 400 MHz; [d] values in parentheses represent coupling constants in Hz.

Table 2. *In vitro* quantitative inhibition of butyrylcholinesterase.

Compound	$IC_{50} \pm$ S.E.M[a] [μM]
1	39.9 ± 0.6
2	299.1 ± 2.0
Galanthamine[b]	21.1 ± 0.01
Tacrine[c]	$0.3 \times 10^{-4} \pm 0.1 \times 10^{-6}$
Eserine[d]	0.85 ± 0.008

[a] Standard mean error of three assays; [b–d] standard inhibitor of butyrylcholinesterase.

Me-H_2O], 422 (21) [M^+-2H_2O], 318 (13) [M^+-ring A], 300 (14) [$C_{21}H_{34}O_2{}^+$-H_2O], 297 (85) [M^+-18-side chain], 175 [$C_{21}H_{34}O_2{}^+$-$C_8H_{15}O_2$]. – HREIMS: $m/z = 458.3842$ (calcd. 458.3762 for $C_{30}H_{48}O_3$).

The compounds **3** and **4** could be identified as 3, 4-epoxyguaia-1(10),11(13)-dien-6,12-olide and 5-hydroxy-7,8,2',3'-tetramethoxy flavone through comparison of physical and spectral data with those in literature [4, 5].

In vitro Butyrylcholinesterase Inhibition Assay. – Horse-serum butyrylcholinesterse (E.C 3.1.1.8), butyrylthiocholine chloride, 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB) galanthamine, tacrine and eserine [(-)-physostigmine] were purchased from Sigma (St. Louis, MO, USA). Buffer and other chemicals were of analytical grade. Butyrylcholinesterase inhibition activity was measured by slightly modified spectrophotometric method developed by Ellman *et al.* [11]. In this assay protocol 150 μl of (100 mM) sodium phosphate buffer (pH 8.0), 10 μl of DTNB, 10 μl of test-compound solution and 20 μl of butyrylcholinesterase solution were mixed and incubated for 15 min (25 °C). The reaction was then initiated by the addition of 10 μl butyrylthiocholine (substrate). The hydrolysis of butyrylthiocholine was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of butyrylthiocholine at a wavelength of 412 nm (15 min). Test compounds and the control were dissolved in MeOH. All the reactions were performed in triplicate in 96-well micro-plate in SpectraMax 340 (Molecular Devices, USA).

Estimation of IC_{50} values. – The concentrations of test compounds that inhibited the hydrolysis of substrate (butyrylthiocholine) by 50% (IC_{50}) was determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The IC_{50} values were then calculated using with the EZ-Fit Enzyme Kinetics program (*Perrella Scientific Inc., Amherst*, USA).

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