Tyrosinase-Inhibitory Long-Chain Esters from *Amberboa ramosa*

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Long-chain esters 1 and 2 have been isolated from the chloroform-soluble fraction of *Amberboa ramosa* and their structures assigned to be methyl 2β(2*S*)-hydroxyl-7(*E*)-tritriacontenoate (1) and methyl 2β(2*S*)-*O*-β-D-galactopyranosyl-7(*E*)-tetratriacontenoate (2). In addition, tricontane (3) and apigenin (4) are also reported for the first time from this species. The structures were assigned on the basis of 1D and 2D NMR techniques. Compounds 1 and 2 showed strong to moderate inhibitory activity against tyrosinase.

Key words *Amberboa ramosa*; Compositae; long-chain ester; tyrosinase inhibition

The genus *Amberboa* belongs to the family Compositae and comprises six species. One of these 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 triterpenoids, flavanoids, steroids, and steroidal glycosides have been reported from this species.[1,2] Here we report the isolation and structure elucidation of long-chain esters **1** and **2**, along with two known compounds tricontane (**3**)[3] and apigenin (**4**).[4]

Tyrosinase (EC 1.14.18.1) is a multifunctional copper-containing enzyme widely distributed in plants and animals. It catalyzes the oxidation of monophenols, *o*-diphenols, and *o*-quinones. Tyrosinase is known to be a key enzyme for melanin biosynthesis in plants and animals. Tyrosinase inhibitors therefore can be clinically useful for the treatment of some dermatologic disorders associated with melanin hyperpigmentation. They also find use in cosmetics for whitening and depigmentation after sunburn. It has recently been shown that other factors such as metal ions and the TRP-1 and TRP-2 enzymes also contribute to the production of melanin. However, tyrosinase plays a critical regulatory role in melanin biosynthesis. Therefore many tyrosinase inhibitors that suppress melanogenesis have been actively studied with the aim of developing preparations for the treatment of hyperpigmentation.[5] Compounds **1** and **2** showed strong to moderate inhibitory activity against tyrosinase.

Results and Discussion

The chloroform-soluble fraction of the whole plant of *A. ramosa* was subjected to column chromatography over flash silica eluted with different mobile phases. Compounds **1**—**4** were finally obtained and their structures established by IR, mass, and NMR spectroscopy including 2D NMR techniques. Compound **1** was isolated as a colorless powder. The molecular formula $C_{34}H_{66}O_3$ was determined based on the HR-EI-MS, FD, and ^{13}C-NMR spectra. HR-EI-MS showed a molecular ion peak at *m*/*z* 522.5017 (Calcd for $C_{34}H_{66}O_3$ 522.5012), suggesting the molecular formula $C_{34}H_{66}O_3$. It exhibited IR absorption bands at 3359, 2920, 2851, 1623, 1466, 1265, 1066, and 966 cm^{-1}. The mass spectrum displayed a strong peak at *m*/*z* 463 for $[M-COOCH_3]^+$ together with a significant peak at *m*/*z* 90 due to McLafferty rearrangement. The fragments at *m*/*z* 145 $[M-C_{27}H_{53}]^+$, 171 $[M-C_{25}H_{51}]^+$, and 351 $[M-C_7H_{11}OHCOOCH_3]^+$ indicated a double bond at C-7. The losses of 31 and 29 mass units from the $[M]^+$ peak were due to the elimination of methoxy and ethyl groups, respectively. The position of the double bond was also confirmed by oxidative cleavage with osmium tetraoxide which gave a mixture of products, among which hexacosanal could be isolated and identified. The ^{1}H-NMR spectrum displayed a triplet at δ 0.85 (J=7.0 Hz) and a broad singlet at δ 1.23 typical of a straight-chain hydrocarbon. It also showed signals at δ 5.40 (1H, dt, J=16.3, 6.1 Hz), 5.37 (1H, dt, J=16.3, 6.1 Hz), 3.94 (m), and 3.85 (br s) for olefinic protons, αH (H-2), and methoxy protons, respectively. The ^{13}C-NMR spectrum (BB and DEPT) showed signals at δ 175.6, 130.7, and 129.6 which could be assigned to the carbonyl and olefinic carbons, respectively. One oxygenated methine and a methoxy carbon resonated at δ 72.2 and 51.4, respectively. The signals observed between 29.0—29.6 indicated the presence of a long chain. The stereochemistry of the double bond was determined based on IR and ^{13}C-NMR spectra. The IR spectrum showed an absorbtion band at 966 cm^{-1} which indicated the *trans* stereochemistry of the double bond.[6] The ^{13}C-NMR spectrum showed allylic carbons in the chain at δ 32.4, typical of methylenes adjacent to a *trans* double bond.[7] The position of the hydroxyl group and the double bond was also confirmed by the HMBC experiments, of which the important correlations are illustrated in Fig. 2.

The optical rotation of **1** was $-120°$, allowing us to assign the S-($-$) configuration to the C-2 chiral center.[8—10] The absolute configuration at C-2 was also established by Horeau's procedure[11,12] (see the Experimental Section). The structure of **1** could be assigned to be methyl 2β(2*S*)-hy-

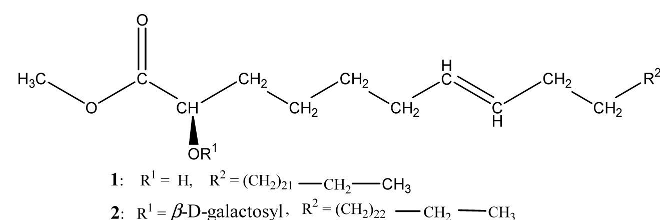

Fig. 1. Structures of Compounds **1** and **2**

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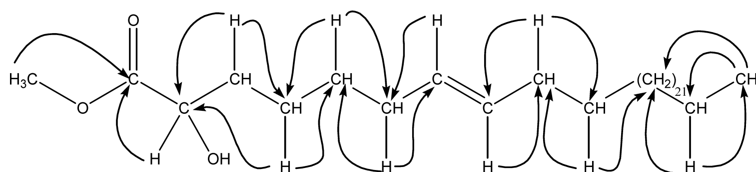
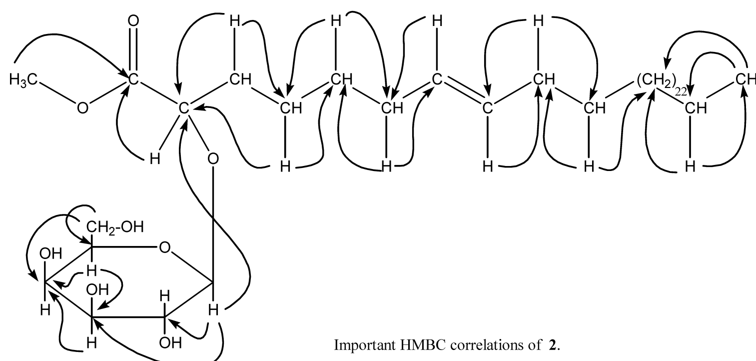


Important HMBC correlations of **1**.



Important HMBC correlations of **2**.

Fig. 2. Important HMBC Correlations of Compounds **1** and **2**

droxyl-7(*E*)-tritriacontenoate.

Compound **2** was isolated as a colorless powder. It showed a molecular ion peak in HR-EI-MS at *m/z* 698.5691, corresponding to the molecular formula $C_{41}H_{78}O_8$ (Calcd for $C_{41}H_{78}O_8$ 699.5697). The IR, UV, ^{1}H- and ^{13}C-NMR spectra of **2** were almost identical to those of **1**, except for additional resonances due to the hexose moiety at δ 4.21 (1H, d, J=7.7 Hz, H-1′), 3.54 (1H, m, H-2′), 3.08 (1H, m, H-3′), 3.84 (1H, m, H-4′), and 3.16 (1H, m, H-5′) and a pair of double doublets at δ 3.60 (1H, J=12.0, 5.7 Hz, H-6′a) and 3.35 (1H, J=12.0, 4.1 Hz, H-6′b) in the ^{1}H-NMR and at δ 102.9, 72.0, 73.2, 71.2, 75.2, and 61.1 in the ^{13}C-NMR spectra. The large coupling constant of the anomeric proton signal at δ 4.21 (d, J=7.7 Hz) confirmed the β-glycoside linkage. The sugar moiety could be identified as galactose by comparing its ^{1}H and ^{13}C chemical shifts with those reported in the literature[13] and further confirmed through acid hydrolysis of **2** which provided various products, among which the glycone could be separated and identified as D-galactose by comparing the retention time of its trimethylsilyl (TMS) ether with that of the standard in gas chromatography (GC) and the sign of its optical rotation. The structure was also confirmed through HMBC correlations, which are illustrated in Fig. 2. On the basis of this evidence, the structure of **2** could be elucidated to be methyl 2β(2*S*)-*O*-β-D-galactopyranosyl-7(*E*)-tetratriacontenoate.

Tyrosinase inhibition studies on the long-chain esters **1** and **2** were carried out. Compound **1**, containing one –OH group at the C-2 position, exhibited highly potent (IC_{50}=1.36 μM) inhibition against the enzyme tyrosinase compared with the standard Kojic acid (IC_{50}=16.67 μM and L-mimosine (IC_{50}=3.68 μM). On the other hand, compound **2**, which contains a D-galactopyranosyl moiety at C-2, also exhibited potent (IC_{50}=11.68 μM) inhibition against tyrosinase compared with Kojic acid (IC_{50}=16.67 μM), but was less potent than compound **1**. This may be due to the presence of the bulky D-galactopyranosyl moiety, which possibly interferes with the entrance of the molecule into the active site of the enzyme tyrosinase. The active site of the tyrosinase contains multifunctional copper molecules that chelate mainly –OH groups. It catalyzes the oxidation of monophenols, *o*-diphenols, and *o*-quinones.[14] None of these

Table 1. ^{13}C-NMR Data of Compounds **1** ($CDCl_3$) and **2** (CD_3OD)

Position	**1** ^{13}C (DEPT)	**2** ^{13}C (DEPT)
1	175.6 (C)	175.6 (C)
2	72.2 (CH)	73.5 (CH)
3	34.3 (CH_2)	34.3 (CH_2)
4	25.6 (CH_2)	25.1 (CH_2)
5	25.1 (CH_2)	25.6 (CH_2)
6	32.5 (CH_2)	32.4 (CH_2)
7	130.7 (CH)	130.5 (CH)
8	129.6 (CH)	129.8 (CH)
9	32.4 (CH_2)	32.4 (CH_2)
10—25	29.6 (CH_2)	29.6 (CH_2)
26	29.5 (CH_2)[a]	29.5 (CH_2)[a]
27	29.4 (CH_2)[a]	29.4 (CH_2)[a]
28	29.3 (CH_2)[a]	29.3 (CH_2)[a]
29	29.2 (CH_2)[a]	29.2 (CH_2)[a]
30	29.0 (CH_2)[a]	29.0 (CH_2)[a]
31	31.7 (CH_2)	29.0 (CH_2)[a]
32	22.5 (CH_2)	31.8 (CH_2)
33	13.9 (CH_3)	22.6 (CH_2)
34	—	13.9 (CH_3)
OCH_3	51.4 (CH_3)	52.9 (CH_3)
1′	—	102.9 (CH)
2′	—	75.2 (CH)
3′	—	73.2 (CH)
4′	—	72.0 (CH)
5′	—	71.2 (CH)
6′	—	61.1 (CH_2)

a) These signals bave been assigned on the basis of literature[17] but may, however, be interchanged.

Table 2. Tyrosinase Inhibitory Activities of the Esters from *Amberboa ramosa*, as Compared with the Standard Inhibitors

Compounds	$IC_{50}\pm$S.E.M.[a] (in μM)
1	1.36±0.1235
2	11.68±0.4456
Kojic acid[b]	16.67±0.519
L-mimosine[b]	3.68±0.02234

a) S.E.M. is the standard error of the mean; *b*) are the standard inhibitors of the enzyme tyrosinase.

compounds showed inhibitory activity against butyrylcholinesterase and ureases (jack bean urease and *Bacillus pasteurii* urease) in 1 mM concentration.

From this study, it can be concluded that the long-chain esters **1** and **2** can be effective inhibitors of tyrosinase enzyme and have the potential to be used for the treatment of hyperpigmentation associated with the high production of melanocytes.

Experimental

General Optical rotations were measured on a JASCO DIP-360 polarimeter. IR spectra were recorded on a 460 Shimadzu spectrometer. EI-MS and HR-FAB-MS were recorded on a 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 MHz for ^{13}C-NMR. 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 The whole plant of *A. ramosa* (Compositae) was collected in June 2002 in Karachi, Pakistan, and identified by Dr. Surraiya Khatoon, Plant Taxonomist, Department of Botany, University of Karachi, where a voucher specimen has been deposited.

Extraction and Isolation The shade-dried plant material (20 kg) was extracted three times with methanol. The residue from the methanolic extract was partitioned between *n*-hexane and water. The water-soluble fraction was further extracted with chloroform, ethylacetate, and *n*-butanol. The chloroform-soluble fraction (55 g) was subjected to column chromatography over flash silica eluted with *n*-hexane–ethylacetate, ethylacetate, and ethylacetate–methanol in increasing order of polarity. The fractions obtained from *n*-hexane–ethylacetate (4 : 1) were combined and further subjected to column chromatography using *n*-hexane–ethylacetate (19 : 1) as the eluent to afford the pure compound **3** (18 mg). The fraction eluted with *n*-hexane–ethylacetate (4 : 1) showed three major and two minor spots on TLC. It was further subjected to column chromatography using *n*-hexane–ethylacetate (8.8 : 1.2) as the eluent to afford pure compounds **1** (15 mg) and **4** (30 mg). The fraction with *n*-hexane–ethylacetate (5.5 : 4.5) was further subjected to column chromatography using *n*-hexane–ethylacetate (6 : 4) as the eluent to afford the pure compound **2** (20 mg).

Compound (**1**): Colorless powder. $[\alpha]_D^{25}$ −120° (c=0.01, $CDCl_3$). IR (KBr) ν_{max} cm^{-1}: 3359, 2920, 2851, 1623, 1466, 1265, 1066, 966. HR-EI-MS *m/z* 522.5017 [M]$^+$ (Calcd for $C_{34}H_{66}O_3$ 522.5012), EI-MS *m/z*: 463 [M−$COOCH_3$]$^+$, 405 [M−$C_3H_5OHCOOCH_3$]$^+$, 351 [M−C_7H_{11}-$OHCOOCH_3$]$^+$, 171 [M−$C_{25}H_{51}$]$^+$, 145 [M−$C_{27}H_{53}$]$^+$, 117 [M−$C_{29}H_{57}$]$^+$, 90, 43, 29. ^{1}H-NMR (400 Mz, $CDCl_3$), δ: 5.40 (1H, dt, J=16.3, 6.1 Hz, H-7), 5.37 (1H, dt, J=16.3, 6.1 Hz, H-8), 3.94 (m, H-2), 3.85 (br s, OMe), 1.94 (m, H-6), 1.78 (m, H-9), 1.58 (m, H-3), 1.50 (m, H-4), 1.40 (m, H-5), 1.23 (br s, 44H, H-10 to H-31) 1.21 (m, H-32), 0.85 (t, J=7.0 Hz, H-33). ^{13}C-NMR data, see Table 1.

Compound (**2**): Colorless powder. $[\alpha]_D^{25}$ −45° (c=0.04, CD_3OD). IR (KBr) ν_{max} cm^{-1}: 3359, 2920, 2851, 1623, 1466, 1265, 1066, 965. HR-EI-MS *m/z* 698.5691 [M]$^+$ (Calcd for $C_{41}H_{78}O_8$ 698.5697), EI-MS *m/z*: 536 [M−sugar]$^+$, 477 [M−sugar−$COOCH_3$]$^+$, 419 [M−sugar−$C_3H_5OHCOOCH_3$]$^+$, 391 [M−sugar−$C_5H_9OHCOOCH_3$]$^+$, 365 [M−sugar−C_7H_{11}-$OHCOOCH_3$]$^+$, 334 [M−sugar−$C_{26}H_{53}$]$^+$, 308 [M−sugar−$C_{28}H_{55}$]$^+$, 162 [sugar]$^+$, 59, 31, 29. ^{1}H-NMR (400 Mz, CD_3OD), δ 5.27 (1H, dt, J=16.4, 6.4 Hz, H-7), 5.24 (1H, dt, J=16.4, 6.4 Hz, H-8), 4.0 (t, J=6.8 Hz, H-2), 4.21 (1H, d, J=7.7 Hz, H-1′), 3.54 (1H, m, H-2′), 3.08 (1H, m, H-3′), 3.84 (1H, m, H-4′), 3.16 (1H, m, H-5′) and 3.60 (1H, dd, J=12.0, 5.7 Hz, H-6′a), 3.35 (1H, dd, J=12.0, 4.1 Hz, H-6′b), 1.94 (m, H-6), 1.78 (m, H-9), 1.58 (m, H-3), 1.50 (m, H-4), 1.40 (m, H-5), 1.23 (br s, 46H, H-10 to H-32) 1.21 (m, H-33), 0.85 (t, J=7.0 Hz, H-34). ^{13}C-NMR data, see Table 1.

Compounds **3** and **4** could be identified as tricontane and apigenin through comparison of their physical and spectral data with those in the literature.[2,4]

Oxidative Cleavage of 1 A solution of **1** (1 mg) in dioxane (0.25 ml) and H_2O (0.15 ml) was treated with OsO_4 (0.3 mg), followed by the addition of the $NaIO_4$ (2.5 mg) at 25 °C. After stirring for 2 h at room temperature, the mixture was concentrated using a Sep-Pak diol cartridge (water). The sample was then eluted with Et_2O. It crystallized from methanol, mp 72°, and gave a molecular ion peak in HR-EI-MS at *m/z* 380.4016 (Calcd 380.4018). It could be identified as hexacosanal by comparison of the melting point and spectral data with those in the literature.[15]

Horeau's Procedure The sample compound (3 mg, *ca.* 0.0091 mmol) was added to a solution of racemic 2-phenylbutanoic anhydride, (0.08 ml) in 0.4 ml of pyridine. The resulting mixture was stirred overnight at room temperature. Distilled water (0.3 ml) was added and the reaction mixture was allowed to stand for 30 min. NaOH (0.1 M) was then added dropwise until the pH became 9, and the solution was then extracted with CH_2Cl_2. The aqueous layer was acidified to pH 3 using HCl 1 M and the acidic layer extracted with C_6H_6 (6 ml). The benzene extract was concentrated to about 2.5 ml. The optical rotation of the resulting 2-phenylbutanoic acid in solution was found to be +85°, thereby establishing the *S*-(−) configuration of the hydroxyl group at C-2 in compound **1**. It was further confirmed by the modified Horeau's procedure in which 4 mg of **1** was dissolved in 12 μl of dry pyridine and 12 μl of racemic 2-phenylbutanoic anhydride. The reaction mixture was kept at 40 °C. After 2 h, 12 μl of (+)-(*R*)-α-phenylethylamine was added and the reaction mixture was stirred. After standing at room temperature for 15 min, 400 ml of ethyl acetate was added and a 1-μl aliquot of the ethyl acetate extract was subjected to GLC analysis (column, OV-17, 2 m×3 mm, 215 °C). The retention time of the resulting amides corresponded to those of standards prepared from 2-phenylbutanoic acid.

Acid Hydrolysis of Compound 2 A solution of **2** (8 mg) in MeOH (5 ml) containing 1 N HCl (4 ml) was refluxed for 4 h, concentrated under reduced pressure, and diluted with H_2O (8 ml). It was extracted with EtOAc and the residue recovered from the organic phase was found to be an inseperable mixture of products. The aqueous phase was concentrated and d-galactose was identified by the sign of its optical rotation ($[\alpha]_D^{25}$ +80.1°). It was also confirmed based on the retention time of its TMS ether (α-anomer 3.8 min, β-anomer 5.2 min) compared with the standard.

Tyrosinase Inhibition Assay Tyrosinase inhibition assays were performed in the 96-well microplate format using a SpectraMax 340 microplate reader (Molecular Devices, CA, U.S.A.) according to the method developed by Hearing.[16] Briefly, first the compounds were screened for the *o*-diphenolase inhibitory activity of tyrosinase using L-DOPA as the substrate. All the active inhibitors from the preliminary screening were subjected to IC_{50} studies. Compounds were dissolved in methanol to a concentration of 2.5%. Thirty units of mushroom tyrosinase (28 nM) were preincubated with the compounds in Na-phosphate buffer 50 nM (pH 6.8) for 10 min at 25 °C. Then L-DOPA (0.5 mM) was added to the reaction mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm (at 37 °C) due to the formation of DOPAchrome for 10 min. The percent inhibition of the enzyme was calculated as follows, using the MS Excel 2000 (Microsoft Corp., U.S.A.)-based program developed for this purpose:

$$\text{percent inhibition (\%)} = [(B-S)/B] \times 100$$

where B and S are the absorbances for the blank and samples, respectively. After screening of the compounds, the median inhibitory concentration (IC_{50}) was also calculated. All the studies were carried out at least in triplicate and the results are expressed as the mean±S.E.M. Kojic acid and L-mimosine were used as standard inhibitors of tyrosinase. All the chemicals and reagents were purchased from Sigma Chemical Co., (St. Louis, MO, U.S.A.).

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