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A new tyrosinase inhibitor from *Crinum yemense* as potential treatment for hyperpigmentation

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A new pyran derivative, 6-hydroxy-2*H*-pyran-3-carbaldehyde, has been isolated from the methanolic extract of *Crinum yemense* along with the known alkaloid haemanthamine, benzoic acid and 1,1′-bis(1,1′-carboxyethyl) ether which is isolated from a natural source for the first time. Structure elucidation of these compounds based on spectroscopic evidences. The pyran derivative was found to be a tyrosinase inhibitor more potent than kojic acid.

1. Introduction

The genus *Crinum* which belongs to the Amaryllidaceae has attracted considerable attention due to its alkaloidal content (Likhitwitayawuid et al. 1993; Abdel-Halim et al. 2004). Besides its popularity as an ornamental garden plant with beautiful blossoms, the plant has a wide range of biological activities as antitumor, immunostimulant, analgesic, antiviral and antimicrobial (Tram et al. 2002). *Crinum yemense* Delflers ex Schweinf. (syn. *C. album* Forssk.) is growing in Yemen and neighboring countries (Wood 1997). In the course of our characterization studies on natural medicines (Abdel-Halim et al. 2004), further isolation and structural elucidation of nitrogenous and non-nitrogenous constituents of the methanolic extract of *C. yemense* are reported. The present study was undertaken to search the plant as a source of natural tyrosinase inhibitors which are used in depigmentation drugs and whitening cosmetics. The present work deals with the isolation of two new compounds, 6-hydroxy-2*H*-pyran-3-carbaldehyde and and 1,1′-bis(1,1′-carboxyethyl) ether, together with benzoic acid and the known alkaloid haemanthamine as well as the tyrosinase inhibitory activity of the pyran derivative. Tyrosinase (EC 1.14.18.1) which belongs to a protein family having the catalytic centre formed by dinuclear copper (type 3 copper protein family), catalyses the orthohydroxylation of monophenol, tyrosine, (cresolase activity) and the subsequent oxidation of the diphenolic product, L-dihydroxyphenylalanine (catecholase activity) to quinone which is a reactive precursor for the synthesis of melanin (Solomon et al. 1996). Tyrosinase is widely distributed in nature in organisms ranging from bacteria to mammals. All tyrosinases, including human and fungal, although not showing a high percentage of identity concerning amino acid sequence, have a conserved domain residing in the central copper-binding domain, which is the site of interaction of tyrosinase with both molecular oxygen and its phenolic substrate (Van Gelder et al. 1997). These domains are quite superimposable for all tyrosinases (Matoba et al. 2006). Differences between tyrosinases from different origins reside in overall sequence homology (primary structure of enzyme), domains other than the copper-binding domain and melanosomal location of the enzyme and its secondary structure (Van Gelder et al. 1997). Moreover, human tyrosinase is a highly glycosylated enzyme compared to mushroom tyrosinase (Wichers et al. 1996). Concerning the catalytic activities of tyrosinases from different origin, both fungal (like that of the mushroom *Agaricus bisporus*) and human tyrosinases possess both monophenol- and diphenoloxygenase activity (Wichers et al. 1996).

An *in vitro* assay using mushroom tyrosinase, from the fungus *Agaricus bisporus*, and L-Dopa (L-dihydroxyphenylalanine) as a substrate was used in this study. The pyran derivative was found to have a significant tyrosinase inhibitory activity more potent than kojic acid. Therefore it was found to be potentially an effective inhibitor of melanin synthesis.

2. Investigations, results and discussion

Compound **1** was isolated as an oil in a yield of 0.06% of the dry weight of extract. Its molecular formula was determined as C_6 H_6O_3 ($[M]^+$ m/z 126.0315 calc. for 126.0317) by HREIMS and ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum exhibited six carbons which were discriminated by DEPT experiment into one methylene, most probably oxygenated (δ 57.4), three methines and two quaternaries. The ^1H NMR spectrum exhibited a total of five protons, of which a downfield singlet at δ 9.57, correlated by HMQC to a carbonyl carbon at δ 177.4, was attributed to an aldehydic proton. A pair of aromatic protons at δ 6.51 (d, J = 3.6 Hz) and 7.22 (d, J = 3.6 Hz) assigned for H-4 and H-5, respectively. The other two protons appeared at δ 4.71 (2 H, br.s) and correlated by HMQC to a carbon signal at δ 57.4 suggested an oxygenated carbon. This was supported by IR absorption bands

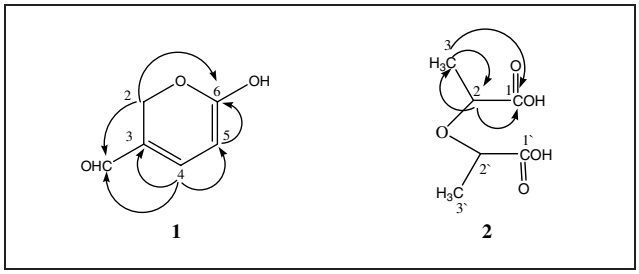

Fig.: HMBC of **1** and **2**

at 1774 cm^{-1} (C=O) and 1192 cm^{-1} (O−C). The positions of the different protons and substituents were corroborated through HMBC spectrum, where cross peaks were observed between H-4 and each of C-5, C-3 and the carbonyl carbon, while both H-2 and H-5 showed correlations to C-6. Thus the structure of **1** was confirmed as 6-hydroxy-2*H*-pyran-3-carbaldehyde, which is a new natural product. It is worth noting that other hydroxyl derivatives of pyran carbaldehydes have been reported before from natural sources (Schulte et al. 1972) but their biological activity was not studied.

Compound **2** was isolated as amorphous solid in a yield of 0.26% of the dry weight of extract. EI MS gave a molecular ion peak at m/z 162 [M^+] which indicated a molecular formula of $C_6H_{10}O_5$. The ^1H NMR spectrum exhibited only two proton signals, a doublet at δ 1.41 (3 H, J = 6.6) and a quartet at δ 4.19 (1 H, J = 6.6), while the ^{13}C NMR spectrum showed three carbon resonances which were discriminated by DEPT experiment into one methyl at δ 21.0, one methine at δ 69.0 and a quaternary carbon at δ 181.5 due to a carboxylic function. This indicated a symmetric molecule. The HMBC spectrum showed cross peaks between the quartet at δ 4.19 and the carbon signals at δ 21.0 and at 181.5 and mutually between the methyl signal at δ 1.41 and the carbon signals at δ 69.0 and 181.5 (Fig.). The collective data of compound **2** led to its identification as 1,1′-bis(1,1′-carboxyethyl) ether, isolated in this study from a natural source for the first time. It is worth noting that symmetric lactic acid dimers and oligomers are well known synthetic products in polymers manufacture (Kenichi et al. 1999; Tencer et al. 1987).

Compound **3** isolated as a crystalline solid was identified as benzoic acid by comparing its spectral data, including ^1H, ^{13}C-NMR and EIMS to published ones (Scott 1972).

Compound **4** was obtained as white needles in a yield of 0.0064% of the dry weight. It was deduced to possess a nitrogen function based on TLC examination using Dragendorff's spray reagent. The physical and spectral data of compound **4** (see Experimental) were found to be in close agreement with those published for (+)haemanthamine (Kobayashi et al. 1977).

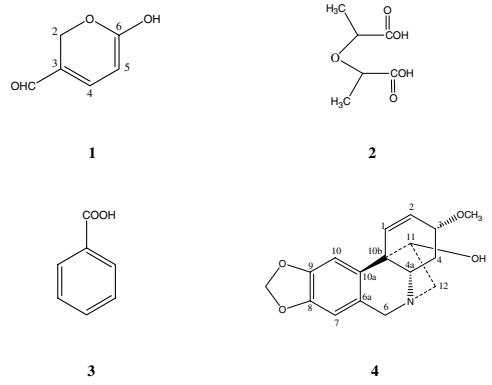

Table: Tyrosinase inhibitory activity of 6-hydroxy-2*H*-pyran-3-carbaldehyde (1)

	Concentration (μM)	Inhibition (%)**	IC_{50} (μM)
Compound 1	3	17.3 ± 1.5	
	10	36.9 ± 2.5	
	30	49.2 ± 3.1	42.2
	50	78.1 ± 5.1	
Kojic acid*	3	27.1 ± 1.8	
	10	48.2 ± 0.9	
	30	57 ± 2.4	75.2
	50	66.3 ± 3.9	

* As positive control
** means ± S.E., n = 4

The isolated pyran derivative, 6-hydroxy-2*H*-pyran-3-carbaldehyde **1**, was tested for Tyrosinase inhibiting activity, based on structural similarity to kojic acid ((5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one). It showed a concentration-dependant reduction in tyrosinase activity (Table) similar to kojic acid in an *in vitro* assay using mushroom tyrosinase and L-dopa as the substrate. It exhibited significant inhibitory effect at 3 μM and higher concentrations with IC_{50} of 42.2 μM. Those values showed that compound **1** is more potent than kojic acid tested in the same assay which showed an IC_{50} value of 75.2 μM. Kojic acid which is frequently used as a positive standard in evaluation of agents inhibitory to tyrosinase activity gave IC_{50} values ranging from 16.7–77.4 μM depending on the assay condition, type of substrate and concentration of the enzyme used in the assay (Sasaki and Yoshizaki 2002; Khan et al. 2006; Kim et al. 2005, 2006). Therefore it was found to be potentially an effective inhibitor of melanin synthesis. It seems interesting that **1** could be used as a skin whitening agent, but further evaluation of its cytotoxicity should be carried out to decide if it could be used safely as a therapeutic agent. It is worth noting that *Crinum yemense* contains another known tyrosinase inhibitor *viz.* benzoic acid which is known to be a copper chelator.

4. Experimental

4.1. Instrumentation

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l = 5 cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; ^1H and ^{13}C NMR spectra, JEOL JNM LA-500 spectrometer (operating at 500 and 125 MHz, respectively), with tetramethylsilane as internal standard; EIMS and HREIMS, JEOL JMS-GCMATE mass spectrometer; HPLC detector, Shimadzu RID-6A refractive index detector. The following experimental conditions were used for chromatography: normal-phase silica gel column chromatography, silica gel BW-200 (Fuji Silysia, 150–300 μm); reversed-phase column chromatography, chromatorex ODS DM 020T (Fuji Silysia, 100–200 μm) and Diion HP-20 (Nippon Rensui); HPLC column, YMC-Pack ODS-A (YMC, 250 × 20 mm i.d); TLC precoated TLC plates with silica gel $60F_{254}$ (Merck, 0.25 mm) (normal phase) and silica gel RP-18 $F_{254\ S}$ (Merck, o.25 mm) (reversed phase); detection was achieved by spraying with Dragendorff's reagent or with 1% Ce $(SO_4)_2-H_2SO_4$ followed by heating; mushroom tyrosinase solution (EC 1.14.18.1), L-dopa and kojic acid (Sigma).

4.2. Plant material

The bulbs of *Crinum yemense* were collected in Ibb province, Yemen, in September 2001 and identified by one of the authors (Abdel-Halim O. B.). A voucher specimen is on file in our laboratory.

4.3. Extraction and isolation

The fresh bulbs of *Crinum yemense* (3.0 kg) were sliced and extracted with 80% MeOH (10L × 4, 80 °C). Evaporation of the solvent under reduced pressure provided the aqueous MeOH extract (301.0 g, 10%). A portion of this extract (250 g) was dissolved in H_2O (ca. 250 mL) and acidified with dilute HCl to pH 2 and then extracted with $CHCl_3$. Removal of the solvent in vacuum yielded the acidic $CHCl_3$-soluble fraction (12 g, 4.8%). The pH of the acidic aqueous solution was adjusted to pH 9 with 28% NH_4OH and then extracted with $CHCl_3$. Removal of the solvent in vacuum yielded the basic $CHCl_3$-soluble fraction (7.0 g, 2.8%). The residue from aqueous layer (231 g) was further extracted with $CHCl_3$-MeOH (3 : 2) to afford 18.5 g (7.4%) residue, which was digested again with $CHCl_3$-MeOH (3 : 2), filtered and left overnight, where **2** deposited as amorphous powder (650 mg, 0.26%). The aqueous layer was further extracted with *n*-BuOH. Evaporation of the solvent afforded 21.0 g residue (8.4%). Separation of the *n*-BuOH extract (21.0 g) on HP 20 Diion (20% MeOH in H_2O) afforded pure **3** (35 mg, 0.014%). The acidic $CHCl_3$-soluble fraction (12 g) was subjected to normal phase silica gel column chromatography (600 g, 45% $CHCl_3$ in *n*-hexane) to afford four fractions. Fraction 2 (1.1 g, 0.44%) was purified on silica gel column chromatography (40 g, 45% $CHCl_3$ in *n*-hexane) to give **1** (152 mg, 0.06%). The basic $CHCl_3$-soluble fraction (3.5 g) was subjected to normal phase silica gel column chromatography [150 g, 40% MeOH in $CHCl_3$-28% NH_4OH (500 : 1)] to afford four fractions. Purification of fraction 2 (1.8, 0.72% g) on reverse phase silica gel open column chromatography (100 g ODS, Chromatorex, 60% MeOH in H_2O) and by HPLC (ODS, 55% MeOH in H_2O) afforded compound **4** (201 mg, 0.08%).

4.4. Tyrosinase inhibitory activity

An *in vitro* assay was performed according to procedures described by Sasaki and Yoshizaki (2002) and reviewed in Atta-Ur-Rahman (2002). Tyrosinase activity using L-dopa as the substrate was assayed spectrophotometrically as follows: 0.1 ml of mushroom tyrosinase solution (EC 1.14.18.1) (625 U/ml) and 0.9 ml of 1/15M phosphate buffered saline (PBS) (buffer solution, 2 mM) were added to 1ml of sample solution dissolved in 5 mM aqueous dimethyl sulfoxide (DMSO), mixed and pre-incubated at 25 °C for 10 min. The reaction was then started by adding 0.03% L-dopa solution. A control reaction (A) was carried out without the test sample and a blank reaction (B) was used for non active heated mushroom tyrosinase. The absorption was measured at 475 nm after incubation for 5 min. Given the sample value as C, the percentage inhibition of tyrosinase activity was calculated as follows: tyrosinase inhibition $\% = (A-C)/(A-B) \times 100$. Kojic acid which is known to be a potent tyrosinase inhibitor (Chen et al. 1991) was used as positive control.

4.5. 6-Hydroxy-2H-pyran-3-carbaldehyde (1)

Oil; UV ν_{max} (MeOH) nm (log ε) 281 (4.15), 255 (3.39); IR ($CHCl_3$, cm^{-1}) 3431 (OH), 1774 (C=O), 1522, 1583 (C=C), 1192 (C=O); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.71 (2 H, br s, H-2), 6.51 (1 H, d, J = 3.6 Hz, H-4), 7.22 (1 H, d, J = 3.6, H-5), 9.57 (1 H, s, H of aldehyde); ^{13}C NMR ($CDCl_3$, 125MHz) δ 57.4 C-2, 109.8 C-4, 122.8 C-5, 152.0 C-3, 160.5 C-6, 177.4 CHO. EIMS 126 [M^+] (48), 109 [M^+-OH] (11), 97 [M^+-CHO] (100), 81 (8), 69 (47), 53 (22); HREIMS m/z 126.0315 (calcd for $C_6H_6O_3$ [M^+], 126.0317).

4.6. 1,1′-Bis(1,1′-carboxyethyl) ether (2)

White amorphous powder, $[\alpha]_D^{24} + 9°$, IR (KBr, cm^{-1}) 1620 (C=O), 1365 (O−CO−CH), 1127 (C−O−C); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.41 (6H, d, J = 6.6 Hz, Me), 4.19 (2 H, q, J = 6.6, H-2, H-2′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.0 (Me), 69.0 (C-2, C-2′), 181.5 (C-1, C-1′), EIMS m/z 162 [M^+] (5), 144 [M^+-H_2O] (100), 126 (18), 115 (15), 103 (46), 91 (22), 86 (58); HREIMS 162.0529 (calcd for $C_6H_{10}O_5$, 162.0525).

4.7. (+)Haemanthamine (4)

Crystalline needles, m.p. 201–203 °C, UV ν_{max} (MeOH) nm (log ε) 293 (3.7), 241 (3.5); $[\alpha]_D^{24} + 20.1°$ (c = 0.31, MeOH); IR (KBr, cm^{-1}) 3350 (OH), 930 (OCH_2O); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.75 (1 H, m, Hβ-4), 2.37 (1 H, m, Hα-4), 3.32 (2 H, m, H_2-12), 3.34 (3 H, s, OMe), 3.60 (1 H, m, H4a), 3.70 (1 H, d, J = 17.0 Hz, Hβ-6), 4.10 (1 H, d, J = 17.0 Hz, Hα-6), 3.79 (1 H, m, H-11), 4.16 (1 H, m, H-3), 5.87 (2 H, br s, OCH_2O), 5.92 (1 H, dd, J = 10.5, 8.5, H-2), 6.25 (1 H, dd, J = 10.5, 2.0, H-1), 6.46 (1 H, s, H-7), 6.78 (1 H, s, H-10); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 30.1 (C-4), 50.1 (C-10b), 55.7 (OCH_3), 61.7 (C-12), 63.4 (C-6), 66.1 (C-4a), 79.2 (C-3), 79.8 (C-11), 100.8 (OCH_2O), 103.1 (C-10), 106.8 (C-7), 123.1 (C-1), 126.5 (C-10b), 135.3 (C-10a), 135.1 (C-2), 146.2 (C-8), 146.5 (C-9); EIMS 301 [M^+] (9), 269 (100), 268 (30), 225 (16), 181 (40), 53 (22).

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