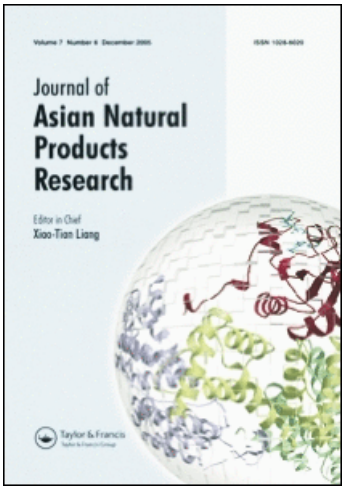

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Asphorodin, a potent lipoxygenase inhibitory triterpene diglycoside from *Asphodelus tenuifolius*

Muhammad Safder[a]; Muhammad Imran[a]; Rashad Mehmood[a]; Abdul Malik[a]; Nighat Afza[b]; Lubna Iqbal[b]; Mehreen Latif[b]
[a] International Centre for Chemical and Biological Sciences, HEJ Research Institute of Chemistry, University of Karachi, Karachi, Pakistan [b] Pharmaceutical Research Center, Karachi, Pakistan

To cite this Article Safder, Muhammad , Imran, Muhammad , Mehmood, Rashad , Malik, Abdul , Afza, Nighat , Iqbal, Lubna and Latif, Mehreen(2009) 'Asphorodin, a potent lipoxygenase inhibitory triterpene diglycoside from *Asphodelus tenuifolius*', Journal of Asian Natural Products Research, 11: 11, 945 — 950
To link to this Article: DOI: 10.1080/10286020903214197
URL: http://dx.doi.org/10.1080/10286020903214197

Journal of Asian Natural Products Research
Vol. 11, No. 11, November 2009, 945–950

Asphorodin, a potent lipoxygenase inhibitory triterpene diglycoside from *Asphodelus tenuifolius*

Muhammad Safder[a], Muhammad Imran[a], Rashad Mehmood[a], Abdul Malik[a]*, Nighat Afza[b], Lubna Iqbal[b] and Mehreen Latif[b]

[a]*International Centre for Chemical and Biological Sciences, HEJ Research Institute of Chemistry, University of Karachi, Karachi 75270, Pakistan;* [b]*Pharmaceutical Research Center, PCSIR Karachi Laboratories Complex, Karachi 75280, Pakistan*

(*Received 18 May 2009; final version received 28 July 2009*)

Asphorodin (**1**), a new diglycoside, has been isolated from the ethyl acetate-soluble fraction of *Asphodelus tenuifolius*. It showed significant inhibitory activity against the enzyme lipoxygenase in a concentration-dependent manner. The Lineweaver–Burk and Dixon plots indicated that the nature of inhibition was non-competitive.

Keywords: *Asphodelus tenuifolius*; Liliaceae; pentacyclic triterpene diglycoside; asphorodin; lipoxygenase inhibition

1. Introduction

The genus *Asphodelus* belongs to the Liliaceae family, which comprises 187 genera and 2500 species. One of the genera of this family is *Asphodelus,* which has seven species distributed in Southern Europe, India, and Pakistan [1,2]. Various species of this genus are used as anti-ulcer and anti-inflammatory agents [3], and also as a diuretic and for the prevention of atherosclerosis [4]. The ethnopharmacological and chemotaxonomic importance of the genus *Asphodelus* prompted us to carry out phytochemical studies on one of its species, namely *Asphodelus tenuifolius*. It is a small erect annual herb, which commonly grows in different areas of Sind and Punjab Provinces of Pakistan [2]. Literature survey revealed that no phytochemical or pharmacological studies have so far been carried out on this species. A methanolic extract of the whole plant showed strong toxicity in the brine shrimp lethality test. On subsequent fractionation, the major toxicity was located in the ethyl acetate-soluble subfraction. Bioassay-directed isolation studies of this fraction have now resulted in the isolation of a new triterpenoidal diglycoside named asphorodin (**1**). Its structure has been elucidated by spectroscopic studies. Compound **1** showed potent inhibitory activity against the enzyme lipoxygenase (LOX) in a concentration-dependent manner. The Lineweaver–Burk and Dixon plots indicated that the nature of inhibition was non-competitive.

2. Results and discussion

The methanolic extract of the whole plant was divided into *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water-soluble fractions. Column chromatography of the ethyl acetate-soluble fraction provided asphorodin (**1**), which gave a positive Liebermann–Burchard test for a pentacyclic

*Corresponding author. Email: abdul.malik@iccs.edu

ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020903214197
http://www.informaworld.com

triterpene and brisk effervescence with dilute sodium bicarbonate, revealing the presence of a carboxylic group. It formed colorless crystals, mp 235–236°C, $[\alpha]_D^{20} + 25$ (MeOH, $c = 1.0$ mg/ml). The IR spectrum showed the presence of a hydroxyl group (3300–3400 cm^{-1}), ester and carboxylic functionalities (1730 cm^{-1}), and an olefinic bond (1650 cm^{-1}). The molecular formula was established as $C_{41}H_{65}O_{14}$ by the positive mode HR-FAB-MS showing the $[M+H]^+$ peak at *m/z* 781.4369. The molecular formula was further supported by the ^{13}C NMR spectrum (BB and DEPT), showing 41 signals including 6 methyl, 11 methylene, 16 methine, and 8 quaternary carbons. Out of these, the two downfield signals at δ 177.7 and 176.7 could be assigned to the ester and carboxylic moieties. The signals of the olefinic carbons resonated at δ 131.4 and 129.4, respectively, while the oxymethine carbon was observed at δ 89.5. The signals of the two anomeric carbons appeared at δ 104.9 and 93.9, while the oxymethylene and oxymethine carbons of the sugar moieties were observed in the range of δ 61.4–76.5. The six methyl groups showed the signals at δ 15.9–27.4. The acid hydrolysis provided a mixture of sugars, which could be identified as D-glucose and L-arabinose by the comparison of retention times of their corresponding tetramethylsilane (TMS) ethers with standards in GC. The aglycone could not be obtained pure due to the paucity of the material. The ^1H NMR spectrum (CD_3OD, 400 MHz) showed anomeric doublets of D-glucose at δ 4.26 (d, $J = 7.8$ Hz) and L-arabinose at δ 5.32 (d, $J = 7.6$ Hz), indicating β- and α-configurations of D-glucose and L-arabinose, respectively. The signals of the olefinic proton at δ 5.56 and the oxymethine proton of the aglycone moiety were observed at δ 3.05 (dd, $J = 11.5$, 4.3 Hz). The oxymethine protons of the sugar units gave signals in the range of δ 3.54–3.73, while the oxymethylene protons were observed in the range of δ 3.33–3.92. Four tertiary methyl groups resonated as singlets at δ 1.19, 1.10, 0.92, 0.85 and two secondary methyl groups gave doublets at δ 1.14 (d, $J = 6.5$ Hz) and 0.71 (d, $J = 6.7$ Hz). The above data corresponded to an ursane-type triterpene. In the EI-MS, the intense fragment ion at *m/z* 604 was due to the $[M^+–CO_2$ Arabinose] peak. Its intensity allowed us to place it at the angular C-17 position, which could also be confirmed by retro-Diels–Alder fragments at *m/z* 410 and 370, indicating the presence of a glucose moiety in rings A/B and a carboxylic group along with an arabinose moiety in rings C/D [5]. The ^1H and ^{13}C NMR spectral data (Table 1) showed a close resemblance to quinovic acid [6] except the downfield shifts of both H-3 and its corresponding carbon. Thus, asphorodin (**1**) is derived from quinovic acid through glycosidations of the hydroxyl group at C-3 and the carboxylic group at C-17 [7–9]. It could be confirmed by HMBC correlations, in which the anomeric proton of the glucose moiety at δ 4.26 (d, $J = 7.8$ Hz) showed ^3J correlation with C-3 and the axial and α-oriented proton of C-3 at δ 3.05 showed ^3J correlations with the anomeric carbon (δ 104.9) of the glucose moiety, as well as with C-1 (δ 38.3) and both the methyl groups at C-4. The anomeric proton of the arabinosyl moiety showed ^3J correlation with the carbonyl group (δ 177.7). On the basis of these evidences, the structure of asphorodin (**1**) could be assigned as 3-*O*-β-D-glucopyranosyl-urs-12-ene-27,28-dioic acid 28-α-L-arabinopyranosyl ester (Figure 1).

Compound **1** inhibited the LOX (EC 1.13.11.12) enzyme in a concentration-dependent fashion with an IC_{50} value of 18 μM. The Lineweaver–Burk as well as Dixon plots indicated that the nature of inhibition was purely a non-competitive type, with a K_i value of 14.8 μM (Table 2). The K_i values were calculated in two ways. First, the slopes of each line in the Lineweaver–Burk plot were plotted against different concentrations of compound **1**. Second, K_i was directly measured from the Dixon plot as an intercept on the

Table 1. ^{1}H and ^{13}C NMR data of asphorodin (400 MHz for ^{1}H, 100 MHz for ^{13}C in CD_3OD).

Position	δ_C	δ_H	Position	δ_C	δ_H
1	38.3	0.89, 1.38	23	27.4	1.19 s
2	24.2	1.87, 2.13	24	20.5	1.10 s
3	89.5	3.05 (dd, $J = 11.5$, 4.3 Hz)	25	15.9	0.85 s
4	39.2	–	26	17.8	0.92 s
5	55.1	0.81	27	176.7	–
6	17.9	1.16, 1.37	28	177.7	–
7	36.3	1.65, 1.76	29	16.1	1.14 (d, $J = 6.5$ Hz)
8	38.6	–	30	16.9	0.71 (d, $J = 6.7$ Hz)
9	46.4	2.76		3-*O*-Glc	
10	36.3	–	1	104.9	4.26 (d, $J = 7.8$ Hz)
11	22.4	1.95, 2.06	2	76.2	3.61
12	129.4	5.56 (br s)	3	76.3	3.65
13	131.4	–	4	72.1	3.73
14	55.6	–	5	76.5	3.55
15	24.9	2.03, 2.39	6	61.4	3.88, 3.92
16	25.6	2.12, 2.41		28-*O*-Ara	
17	49.1	–	1	93.9	5.32 (d, $J = 7.6$ Hz)
18	53.5	2.82 (d, $J = 11.2$ Hz)	2	69.9	3.68
19	38.5	1.38	3	75.3	3.61
20	36.6	1.01	4	69.6	3.54
21	29.6	1.17, 1.35	5	61.6	3.33, 3.85
22	35.4	1.52, 1.97			

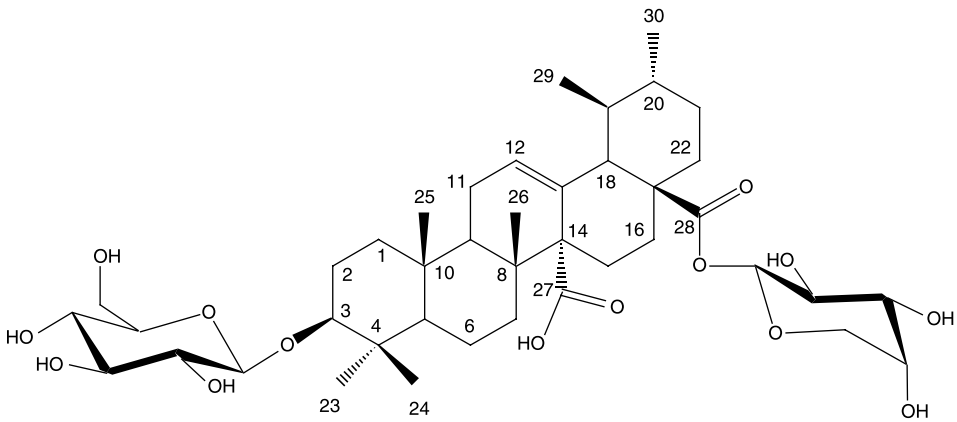

Figure 1. Structure of asphorodin (**1**).

x-axis. Determination of the inhibition type is critical for identifying the mechanism of inhibition and the sites of inhibitor binding. The Lineweaver–Burk and Dixon plots and their replots indicated a pure non-competitive type of inhibition of compound **1** against the LOX enzyme. In other words, we can say that compound **1** and linoleic acid bind randomly and independently at the different sites of LOX. This indicates that the inhibition depends only on the concentrations of compound **1** and the dissociation constant (K_i) [10].

3. Experimental

3.1 General experimental procedures

Melting points were determined using a Gallenkamp apparatus and are uncorrected.

Table 2. *In vitro* LOX inhibition by compound **1**.

S. no.	Compounds	IC_{50} (μM) ± SEM	K_i[a] (μM) ± SEM[b]	Type of inhibition
1	Compound **1**	18.1 ± 0.12	14.2 ± 0.18	Non-competitive
2	Baicalein[c]	22.6 ± 0.08	18.0 ± 0.03	Mixed type

[a]K_i (dissociation constant or inhibition constant) was determined from a nonlinear regression analysis by the Dixon plot and secondary Lineweaver–Burk plot at various concentrations of compound **1** (each point in the Lineweaver–Burk and Dixon plots and their replots represents the mean of three determinations).
[b]Standard mean error of 3–5 assays.
[c]Positive control used in assays.

UV and IR spectra were recorded on Hitachi-UV-3200 and Jasco-320-A spectrometers, respectively. ^1H and ^{13}C NMR spectra were recorded on a Bruker AM-400 spectrometer with TMS as an external standard. The 2D NMR spectra were recorded on a Bruker AMX 400 NMR spectrometer. Optical rotations were measured on a Jasco DIP-360 digital polarimeter using a 10 cm cell tube. Mass spectra (EI and HR-EI-MS) were measured in an electron impact mode on a Finnigan MAT 12 or MAT 312 spectrometer and ions are given in *m/z* (%). TLC was performed with precoated silica gel G-25-UV_{254} plates (20 × 20 cm; E. Merck, Darmstadt, Germany) and detection was done at 254 nm, and by spraying with ceric sulfate in 10% H_2SO_4. Silica gel (230–400 mesh; E. Merck) was used for column chromatography. For enzyme inhibition assay, all chemicals used including LOX were purchased from Sigma Chemical Company (St Louis, MO, USA).

3.2 LOX inhibition assay

LOX-inhibiting activity was measured by modifying the spectrophotometric method developed by Tappel. LOX (1.13.11.12) type I-B (Soybean) and linoleic acid were purchased from Sigma. All other chemicals were of analytical grade. The assay conditions and protocol were the same as described in our previous article. The kinetic study was performed in 96-well microtiter plates using SpectraMax 384plus (Molecular Devices, Sunnyvale, CA, USA). The IC_{50} values were then calculated using the EZ-Fit Enzyme kinetics program.

3.3 Determination of kinetic parameters

Dissociation constant/inhibition constant (K_i) was determined by the interpretation of the Dixon and Lineweaver–Burk plots (Figures 2 and 3) and their secondary replots using initial velocities obtained over a substrate (linoleic acid) concentration range between 0.05 and 0.2 mM. The dependence of V_{max}/K_m and V_{max} is given by

$$V_{max}/K_m = \left(\frac{(V_{max}/K_m)K_1}{K_1 + [1]}\right) \Rightarrow 1$$

$$= \frac{K_1}{K_1 + [1]} \Rightarrow K_1 = K_1 + [1].$$

3.4 Plant material

The whole plant of *A. tenuifolius* Cav. (8 kg) was collected from the Cholistan Desert (Bahawalpur) in May 2006 and identified by Dr Muhammad Arshad, Plant Taxonomist, Cholistan Institute for Desert Studies (CIDS), the Islamia University of Bahawalpur, where a voucher specimen (28/CIDS/06) has been deposited.

3.5 Extraction and isolation

The freshly collected whole plant material (8 kg) was cut into small pieces and extracted with MeOH (3 × 30 liters). The combined methanolic extract was

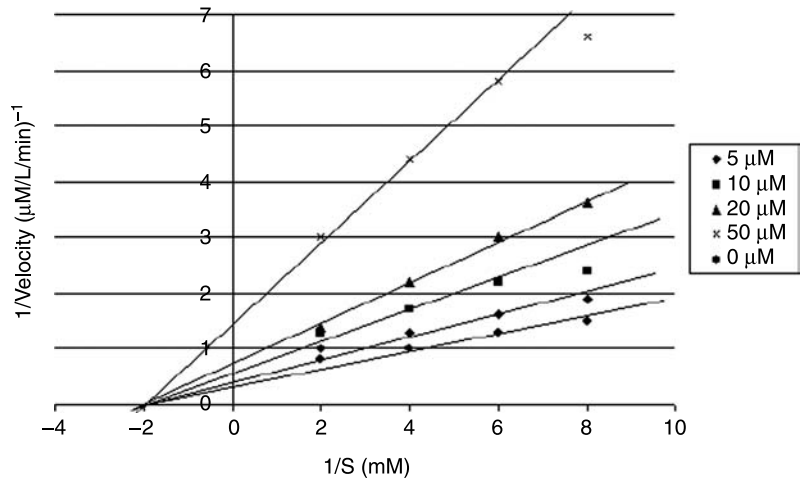

Figure 2. Lineweaver–Burk plot of compound **1** of the reciprocal of initial velocities versus the reciprocal of four fixed linoleic acid (substrate) concentrations in the absence (0 μM) and presence of 5, 10, 20, and 50 μM of compound **1**.

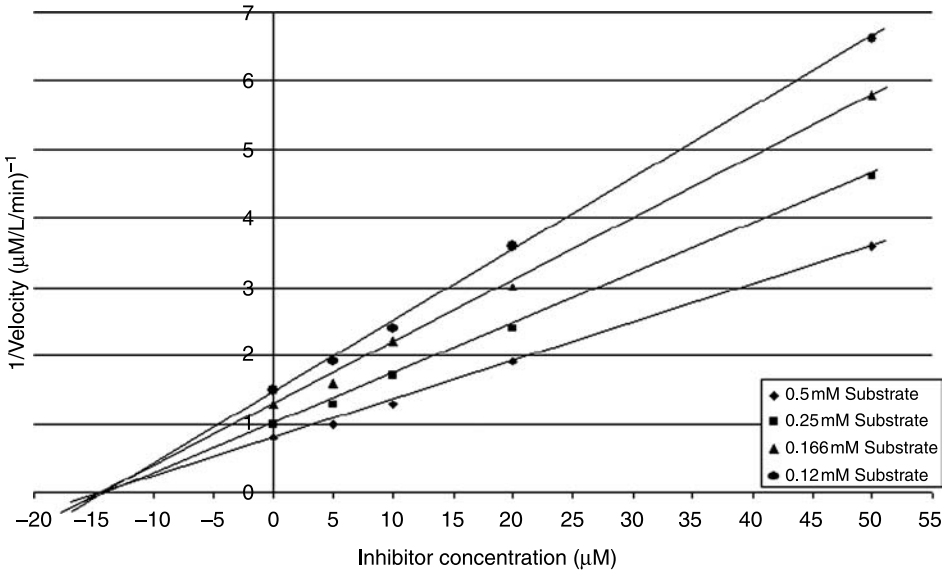

Figure 3. Dixon plot of compound **1** at four fixed linoleic acid concentrations, 0.5, 0.25, 0.16, and 0.12 mM. Each point in the graph represents the mean of three experiments.

evaporated under reduced pressure to yield a residue (300 g), which was divided into *n*-hexane (45 g), $CHCl_3$ (85 g), EtOAc (75 g), *n*-BuOH (45 g), and water (38 g) soluble fractions. The EtOAc-soluble fraction was subjected to column chromatography over silica gel, eluting with $CHCl_3$–MeOH in the increasing order of polarity. The fractions obtained from $CHCl_3$–MeOH (1:1) were combined and rechromatographed over silica gel, eluting with ethyl acetate:MeOH in the increasing order of polarity to afford compound **1** (17 mg).

*3.5.1 Asphorodin (**1**)*

Colorless crystals, mp 235–236°C. $[\alpha]_D^{20} + 25$ (MeOH, $c = 1.0$ mg/ml). IR (KBr) ν_{max}: 3300–3400 cm^{-1} (OH), 1730 cm^{-1} (carbonyl of ester and COOH moieties), 1650 cm^{-1} (C═C), 1085 cm^{-1} (C—O); ^1H and ^{13}C NMR spectral data, see Table 1; HR-FAB-MS: *m/z* 781.4369 $[M+H]^+$ (calcd for $C_{41}H_{65}O_{14}$, 781.4374).

3.6 Acid hydrolysis

A solution of **1** (4 mg) in methanol (5 ml) and 1 M HCl (3 ml) was refluxed for 4 h. The solution was concentrated under reduced pressure, diluted with H_2O (5.5 ml), and, after neutralization, the reaction mixture was concentrated and the residue was passed through a Sep-Pak C18 cartridge with water and methanol. The eluent was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (1.5 mg) in pyridine (1.0 ml) at 60°C for 2 h. After dryness, the residue was treated with *N*-(trimethylsilyl)imidazole (1.0 ml) at 60°C. The supernatant was applied to GC with an OV-17 column, and an H_2 flame ionization detector with a vaporization temperature of 280°C and a detector temperature of 280°C. The R_t of D-glucose and L-arabinose was 13.8 and 7.3 min, respectively [11].

References

[1] T. Cooke, *The Flora of Presidency of Bomboy* (Taylor and Francis Printers, London, 1908), Vol. II, p. 760.
[2] S.J.D. Hooker, *Flora of British India* (Goyal Offset Printers, London, 1894), Vol. 6, p. 332.
[3] S.R. Baquar, *Medicinal and Poisonous Plants of Pakistan*, 1st ed. (Karachi Printing Press, Karachi, 1989), p. 45.
[4] S.Y.R. Chandha, *The Wealth of India*, A dictionary of Indian raw materials and industrial products (CSIR, Niscair Press, New Delhi, 2003), Vol. I, p. 472.
[5] H. Budzikiewicz, J.M. Wilson, and C. Djerassi, *J. Am. Chem. Soc.* **85**, 3688 (1963).
[6] K. Pollmann, S. Gagel, M.H.A. Elgamal, K.H. Shaker, and K. Seifert, *Phytochemistry* **44**, 485 (1997).
[7] G.A. Miana and H.M.G. Al-Hazimi, *Phytochemistry* **26**, 225 (1987).
[8] R. Aquino, F.D. Simone, C. Pizza, R. Cerri, and J.E.D. Mello, *Phytochemistry* **27**, 2927 (1988).
[9] V.U. Ahmad, S. Uddin, S. Bano, and I. Fatima, *Phytochemistry* **28**, 2169 (1989).
[10] I. Ahmad, S. Ahmad, S.A. Nawaz, N. Afza, A. Malik, A. Fatima, S.B. Khan, M. Ahmad, and M.I. Choudhary, *Chem. Pharm. Bull.* **53**, 907 (2005).
[11] A. Ttoh, T. Tanahashi, N. Nagakura, and T. Nishi, *Chem. Pharm. Bull.* **51**, 1335 (2003).