Pakistolides A and B, Novel Enzyme Inhibitory and Antioxidant Dimeric 4-(Glucosyloxy)Benzoates from *Berchemia pakistanica*

by **Naveen Mukhtar**[a]), **Abdul Malik***[a]), **Naheed Riaz**[a]), **Kiran Iqbal**[a]), **Rasool Bakhsh Tareen**[b]), **Shamsun Nahar Khan**[a]), **Sarfraz Ahmad Nawaz**[a]), **Juveria Siddiqui**[a]), and **M. Iqbal Choudhary**[a])

[a]) H.E.J. Research Institute of Chemistry, International Center for Chemical Sciences, University of Karachi, Karachi-75270, Pakistan

[b]) Department of Botany, Baluchistan University, Quetta, Pakistan

(phone: (92-21)-9243198; fax: (92-21)-9243190; e-mail: hejric@digicom.net.pk, hej@digicom.net.pk)

Pakistolides A and B, novel dimeric β-(glucosyloxy)benzoates were isolated from *Berchemia pakistanica* and assigned structures **1** and **2** on the basis of extensive NMR studies. In addition, the known compounds 7,5′-dimethoxy-3,5,2′-trihydroxyflavone (= 3,5-dihydroxy-2-(2-hydroxy-5-methoxyphenyl)-7-methoxy-4*H*-1-benzopyran-4-one), 4′,5-dihydroxy-3,6,7-trimethoxyflavone (= 5-hydroxy-2-(4-hydroxyphenyl)-3,6,7-trimethoxy-4*H*-1-benzopyran-4-one), 5,6-dihydroxy-4,7-dimethoxy-2-methylanthracene-9,10-dione, and 1,3,4-trihydroxy-6,7,8-trimethoxy-2-methylanthracene-9,10-dione were reported for the first time from the genus *Berchemia*. Both **1** and **2** showed significant α-glucosidase and lipoxygenase inhibitory activities, while **2** also showed antioxidant potential.

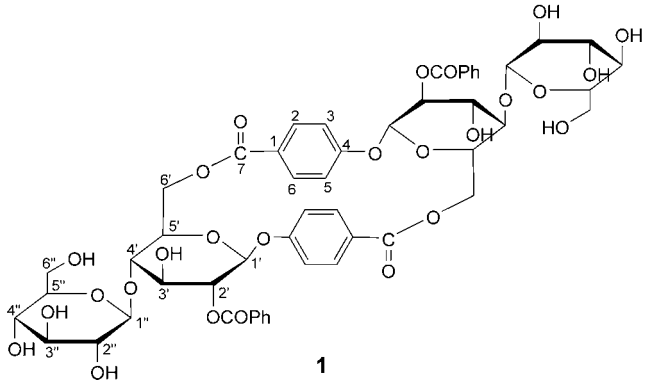
Introduction. – The genus *Berchemia* (Rhamnaceae) comprises *ca.* 10 species scattered throughout the tropics of Asia, Africa, and America. In Pakistan, it is represented by three species, *B. floribunda*, *B. pakistanica*, and *B. edgeworthii* [1]. The roots and stems of *Berchemia* species are used for the treatment of gall stones, liver diseases, neuraglia, and stomach cramp in Japan, and also used in traditional Chinese medicine as an antipyretic, a diuretic, and for the treatment of rheumatism and lumbago [2]. A literature survey revealed that little phytochemical work has been carried out on the genus *Berchemia*, and only some lignans [3], biflavonoids [4][5], benzofuranoid-type flavonoids [6], and derivatives of aromatic compounds [2][7] have so far been reported. *Berchemia pakistanica* BROWICZ. is found only in the Quetta, Ziarat, and Waziristan regions of Pakistan [1]. No pharmacological screening or phytochemical studies have so far been carried out on this indigenous species. The MeOH extract of *B. pakistanica* showed strong cytotoxicity in the brine shrimp lethality test [8]. Further pharmacological screening revealed pronounced α-glucosidase and lipoxygenase inhibitory and antioxidant activities of the AcOEt fraction. This prompted us to carry out bioassay-directed isolation studies of the AcOEt-soluble fraction of *B. pakistanica*. As a result of these studies, we isolated two novel bioactive dimeric 4-(glucosyloxy)benzoates, named pakistolides A (**1**) and B (**2**), along with the known compounds 7,5′-dimethoxy-3,5,2′-trihydroxyflavone (= 3,5-dihydroxy-2-(2-hydroxy-5-methoxyphenyl)-7-methoxy-4*H*-1-benzopyran-4-one) [9], 4′,5-dihydroxy-3,6,7-trimethoxyflavone (= 5-hydroxy-2-(4-hydroxyphenyl)-3,6,7-trimethoxy-4*H*-1-benzopyran-4-one) [10], 5,6-dihydroxy-4,7-dimethoxy-2-methylanthracene-9,10-dione [11], and 1,3,4-trihydroxy-6,7,8-trimethoxy-2-methylanthracene-9,10-dione [12] which are reported for the first time from the genus *Berchemia*.

α-Glucosidase (EC 3.2.1.20) is the general term for the hydrolases of α-glucosidase in the small intestine. α-Glucosidase belongs to the class of hydrolase enzymes, one of the major six classes of enzymes. This class of enzymes can be considered as a special class of the transferases in which the donor group is transferred to water. The generalized reaction involves the hydrolytic cleavage of C–O, C–N, O–P, and C–S bonds. Glycoside hydrolases catalyze the selective hydrolysis of glycosidic bonds in oligosaccharides. α-Glucosidase hydrolyzes both the $1\rightarrow4$ and $1\rightarrow6$ linkages. For example, $(1\rightarrow4)$-α-D-glucosidic oligosaccharides from maltose to maltoheptaose are hydrolyzed to glucose by α-glucosidase; $(1\rightarrow6)$-linked oligo-α-D-saccharides such as panose (the product of the pullulanase II action on pullulan) are also hydrolyzed by α-glucosidase [13]. It plays a physiologically important role in the digestion process of dietary carbohydrates aimed at suppressing postprandial hyperglycemia [14]. Non-insulin-dependent diabetes mellitus is caused by a secretory decrease in insulin from pancreatic Langerhans β cells or lowering of the insulin resistance. In addition, long-term manifestation of this disease can result in the development of retinopathy, neuropathy, cataracts, and so on. Among the therapeutic drugs to prevent a high blood-glucose level, the inhibitors of α-glucosidase, which is a membrane-bound enzyme at the epithelium of the small intestine that catalyzes the cleavage of glucose from disaccharide, are effective for delaying glucose absorption [15]. α-Glucosidase inhibitors such as deoxynojirimycin and *N*-butyldeoxynojirimycin are potent inhibitors of human-immunodeficiency-virus (HIV) replication and HIV-mediated syncytium formation *in vitro* [16]. Inhibition of α-glucosidases causes abnormal functionality of glycoproteins because of incomplete modification of glycans. Suppression of this processing is to be expected for antiviral activity and decrease of growth rate of the tumor [17].

Lipoxygenases (EC 1.13.11.12) constitute a family of non-haem iron-containing dioxygenases that are widely distributed in animals and plants. In mammalian cells, they are key enzymes in the biosynthesis of a variety of bioregulatory compounds such as hydroxyeicosatetraenoic acids (HETEs), leukotrienes, lipoxins, and hepoxylines [18]. These lipoxygenase products play a role in a variety of disorders such as bronchial asthma and inflammation [19]. The arachidonic acid metabolism through lipoxygenase pathways generates various biologically active lipids that play important roles in thrombosis and tumor progressions. Angiogenesis, the formation of new capillary vessels from preexisting ones, underpins a number of physiological processes and participates in the development of several pathological conditions such as arthritis and cancer [20]. Lipoxygenases are, therefore, potential targets for the rational drug design and discovery of mechanism-based inhibitors for the treatment of a variety of disorders such as bronchial asthma, inflammation, cancer, and autoimmune diseases.

There is extensive evidence to implicate radicals in the development of degenerative diseases. It is suggested that radical damage to cells leads to the pathological changes associated with aging. Radicals may also be a contributory factor in a progressive decline in the function of the immune system. The consequences of oxidative stress are serious, and in many cases are manifested by increased activities of enzymes involved in oxygen detoxification. Identification of new antioxidants remains a highly active research area because antioxidants may reduce the risk of various chronic diseases caused by radicals [21].

Result and Discussion. – The HR-FAB-MS (positive-ion mode) of **1** provided $[M+H]^+$ at *m/z* 1097.3124, corresponding to the molecular formula $C_{52}H_{56}O_{26}$, which indicated 25 degrees of unsaturation. The UV spectrum exhibited λ_{max} at 216, 255, and 296 nm, typical for the presence of aromatic rings. The IR spectrum showed strong absorptions at 3365 (O–H), 2925 (C–H), 1652 (C=O), 1603, 1517 (aromatic C=C), and 1220 (C–O) cm^{-1}. The dimeric nature of **1** was revealed by a strong peak at *m/z* 549.1595 ($C_{26}H_{29}O_{13}^+$) in the HR-FAB-MS (positive-ion mode). Further mass fragments originated from this peak, which was evident by link scan measurements. Besides the peak at *m/z* 531.1491 ($C_{26}H_{27}O_{12}^+$) due to the loss of H_2O, it also showed prominent peaks at *m/z* 387.1061 ($C_{20}H_{19}O_8^+$) and 282.0723 ($C_{13}H_{14}O_7^+$) due to the loss of glucose and monobenzoylated glucose moieties, respectively. In view of the spectral data and comparison with berchemolide [7], the structure **1** was assigned to pakistolide A, which was further supported by HMQC as well as HMBC experiments (*Table 1*).



1

In the ^{1}H-NMR spectrum (*Table 1*) of **1**, the two 1,4-disubstituted benzene rings could be inferred by a set of *ortho*-coupled H-atoms appearing as *d* at δ 7.17 ($J = 7.5$ Hz) and 6.91 ($J = 7.5$ Hz) integrating for 8 H-atoms. The presence of two further monosubstituted benzene rings were evident by a *m* (10 H) at δ 7.36–7.98. The presence of four sugar moieties in β-D-configuration was revealed by the *d* of anomeric protons at δ 5.09 (2 H) and 4.72 (2 H). The H-atoms geminal to OH groups resonated at δ 3.46–3.64 (*m*, 12 H). The H-atoms geminal to the benzoyloxy groups appeared downfield at δ 5.25 (*t*, $J = 7.9$ Hz, 2 H). Two sets of *AB* '*dd*' integrating for two protons each at δ 4.65 ($J = 11.5, 2.1$ Hz) and 4.40 ($J = 11.5, 7.9$ Hz) and δ 3.82 ($J = 10.4, 1.8$ Hz) and 3.66 ($J =$ 10.4, 5.5 Hz) were due to the C*H*$_2$OH groups of the four hexose moieties. Upon rationalizing these data, it is evident that pakistolide A is a dimer of a 4-hydroxybenzoate derivative. This was confirmed by the ^{13}C-NMR spectrum (BB, DEPT; *Table 1*) of **1**, which showed resonance for 22 C-atoms comprising 2 CH_2, 15 CH, and 5 quaternary C-atoms. However, signals of some C-atoms were very intense due to overlapping resulting from the symmetry of the molecule. The downfield signals at δ 166.7 and 166.2 indicated the presence of two ester moieties. Another downfield signal at δ 155.3 was due to O-bearing quatenary C-atoms. The hexose moieties could be identified by comparison of ^{1}H- and ^{13}C-NMR data with literature values [22] and were further confirmed by acid hydrolysis (see the *Exper. Part*), which yielded, besides host of other products, D-glucose, identified by the sign of its optical rotation and comparison of the GC retention time of its trimethylsilyl ether with that of a standard. The benzoyloxy groups were located at C(2′) of two of the four glucose moieties as suggested by the downfield shifts of both H–C(2′) (δ 5.25) and C(2′) (δ 75.1) with respect to glucose. Two further H-atoms geminal to O-atoms were observed rather downfield at δ 4.53 (*m*, 2 H) due to the 1 → 4 linkage of two glucose moieties. The important correlations in the COSY-45° experiment of **1** exhibited coupling between H–C(2′) (δ 5.25) and H–C(1′) (δ 5.09). H–C(4′) at δ 4.53 coupled with H–C(3′) and H–C(5′), which

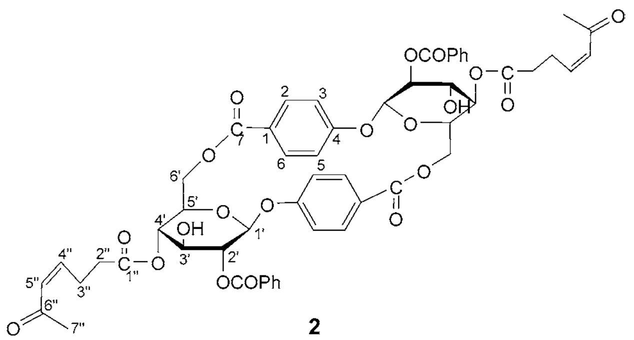
Table 1. *^{1}H- and ^{13}C-NMR Data and Important HMBC Correlations of Compound* **1**. $CD_3OD/CDCl_3$ soln.; δ in ppm, *J* in Hz.

	δ(C)[a])	δ(H)[b])	HMBC[c]) (H → C)
C(1)	123.1 (C)	–	–
H−C(2,6)	129.4 (CH)	7.17 (*d*, *J* = 7.5)	C(7), C(4)
H−C(3,5)	115.2 (CH)	6.91 (*d*, *J* = 7.5)	C(1)
C(4)	155.3 (C)	–	–
C(7)	166.7 (C)	–	–
H−C(1′)	99.6 (CH)	5.09 (*d*, *J* = 7.9)	C(4), C(3′), C(5′)
H−C(2′)	75.1 (CH)	5.25 (*t*, *J* = 7.9)	C(4′)
H−C(3′)	73.0 (CH)	3.58 (*m*)	C(1′), C(5′)
H−C(4′)	75.8 (CH)	4.53 (*m*)	C(1″), C(2′), C(6′)
H−C(5′)	70.0 (CH)	3.60 (*m*)	–
CH_2(6′)	64.0 (CH_2)	4.40 (*dd*, *J* = 11.5, 7.9), 4.65 (*dd*, *J* = 11.5, 2.1)	C(7), C(4′)
H−(1″)	102.3 (CH)	4.72 (*d*, *J* = 7.7)	C(4′), C(3″), C(5″)
H−C(2″)	73.4 (CH)	3.54 (*t*, *J* = 7.7)	C(4″)
H−C(3″)	76.3 (CH)	3.46 (*m*)	C(1″), C(5″)
H−C(4″	70.2 (CH)	3.50 (*m*)	C(6″), C(2″)
H−C(5″)	77.2 (CH)	3.64 (*m*)	C(1″), C(4″), C(3″)
CH_2(6″)	61.2 (CH_2)	3.66 (*dd*, *J* = 10.4, 5.5), 3.82 (*dd*, *J* = 10.4, 1.8)	–

[a]) Recorded at 100 MHz. [b]) Recorded at 400 MHz. [c]) Recorded at 500 MHz.

resonated at δ 3.58 and 3.60, respectively. The CH_2(6′) group at δ 4.40 and 4.65 displayed a cross-peak with H−C(5′), which resonated at δ 3.60.

Compound **2** displayed a $[M + H]^+$ peak at *m/z* 1021.3119 in the HR-FAB-MS (positive-ion mode), corresponding to the molecular formula $C_{54}H_{52}O_{20}$. The peak at *m/z* 511.1590 ($C_{27}H_{27}O_{10}^+$) indicated the dimeric nature of compound **2**. A further ion at *m/z* 406.1250 ($C_{20}H_{22}O_9^+$) was due to the loss of a benzoyl (C_7H_5O) group from the ion at *m/z* 511.1590. The ion at *m/z* 386.0990 ($C_{20}H_{18}O_8^+$) arose by the loss of the side chain ($C_7H_9O_2$) from the ion at *m/z* 511.1590. The peaks at *m/z* 349.1064 ($C_{21}H_{17}O_5^+$) and 244.0725 ($C_{14}H_{12}O_4^+$) were due to the losses of glucose and monobenzoylated glucose moieties. The UV and IR spectra of compound **2** were identical to those of **1**. Several peaks in the ^{1}H- and ^{13}C-NMR spectra (*Table 2*) and HMQC, COSY-45°, and HMBC plots were very similar to those of **1** allowing us to assign structure **2** to pakistolide B.



2

Table 2. *^{1}H- and ^{13}C-NMR Data and Important HMBC Correlations of Compound* **2**. $CD_3OD/CDCl_3$ soln.; δ in ppm, J in Hz.

	δ(C)[a])	δ(H)[b])	HMBC[c]) (H → C)
C(1)	123.5 (C)	–	–
H–C(2,6)	130.1 (CH)	7.02 (d, J = 7.8)	C(7), C(4)
H–C(3,5)	115.5 (CH)	6.82 (d, J = 7.8)	C(1)
C(4)	154.8 (C)	–	–
C(7)	169.5 (C)	–	–
H–C(1′)	99.4 (CH)	4.98 (d, J = 7.9)	C(4), C(3′), C(5′)
H–C(2′)	76.0 (CH)	5.13 (t, J = 7.9)	C(4′)
H–C(3′)	74.5 (CH)	3.62 (m)	C(1′), C(5′)
H–C(4′)	76.8 (CH)	4.93 (m)	C(1″), C(2′), C(6′)
H–C(5′)	70.2 (CH)	3.45 (m)	–
CH_2(6′)	63.8 (CH_2)	4.60 (dd, J = 12.6, 9.0), 4.88 (dd, J = 12.6, 2.2)	C(7), C(4′)
C(1″)	173.0 (C)	–	–
CH_2(2″)	29.6 (CH_2)	2.38 (m)	C(4″)
CH_2(3″)	27.3 (CH_2)	2.36 (m)	C(1″), C(5″)
H–C(4″)	132.1 (CH)	5.93 (dd, J = 9.6, 3.9)	C(6″), C(2″)
H–C(5″)	127.5 (CH)	5.52 (d, J = 9.6)	C(3″), C(7″)
C(6″)	205.9 (C)	–	–
Me(7″)	26.7 (CH_3)	2.33 (s)	C(5″)

[a]) Recorded at 125 MHz. [b]) Recorded at 500 MHz. [c]) Recorded at 500 MHz.

The ^{1}H-NMR spectrum (*Table 2*) of **2** displayed a downfield *dd* at δ 5.93 (J = 9.6, 3.9 Hz, 2 H) and a *d* at δ 5.52 (J = 9.6 Hz, 2 H), indicating the presence of two C=C bonds in (*Z*)-configuration. The presence of two C_7 side chains was evident by signals of 14 H-atoms ranging from δ 2.33–2.38. The ^{13}C-NMR spectrum showed downfield signals at δ 205.9 and 173.0 for the ketone and ester functionalities. Other signals at δ 132.1 and 127.5 were due to the aliphatic C=C bond. The remaining signals of the side chain appeared at δ 29.6 and 27.3 (2 CH_2) and 26.7 (Me). The (*Z*)-configuration of the aliphatic C=C bond was confirmed by the ^{13}C-NMR chemical shift of CH_2C=C at δ(C) 27.3 since δ *ca.* 27 and *ca.* 32 have been reported for the (*Z*)- and (*E*)-type, respectively [23]. The COSY-45° experiment of **2** revealed coupling between H–C(4″) (δ 5.93) and H–C(3″) (δ 2.36) and H–C(5″) (δ 5.52) confirming the location of the C=C bond, it also confirmed the ^2J correlation of Me(7″) (δ 2.33) and C(6″) (δ 205.9) and the ^3J interaction with C(5″) (δ 127.5) as deduced from the HMBC.

The inhibitory activity of **1** and **2** against α-glucosidase was studied (*Figs. 1* and *2*). The IC_{50} values (*Table 3*) showed that compound **1** is very potent while **2** has significant

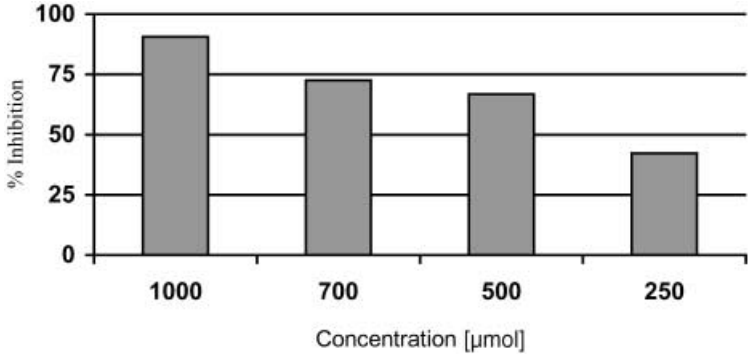


Fig. 1. *Inhibition of α-glucocidase by* **1** *at various concentrations.* For conditions, see *Exper. Part*.

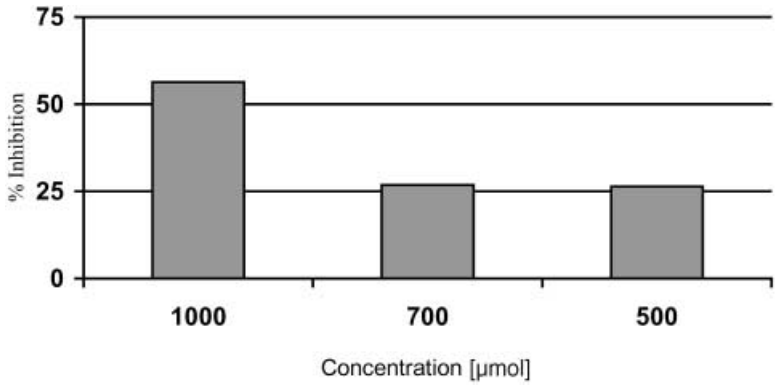


Fig. 2. *Inhibition of α-glucocidase by* **2** *at various concentrations.* For conditions, see *Exper. Part*.

Table 3. *α-Glucosidase and Lipoxygenase Inhibitory Activities of the Compounds* **1** *and* **2** *as Compared with the Standard Inhibitors*

	$IC_{50} \pm$ s.e.m[a]) [µM]	
	α-Glucosidase	Lipoxygenase
1	450 ± 29.5	27.8 ± 0.20
2	937 ± 31.7	41.2 ± 0.50
Deoxynojirimycin[b])	425 ± 8.14	–
Baicalein[c])	–	22.4 ± 0.25

[a]) Standard error of the mean of 3–5 assays. [b]) Standard inhibitor of α-glucosidase. [c]) Standard inhibitor of lipoxygenase.

activity as compared to the standard inhibitor. The stronger action of **1** is probably due to the presence of more glucose units, which enhance the α-glucosidase activity.

Both **1** and **2** were also subjected to a lipoxygenase inhibition assay (*Figs. 3* and *4*). From the IC_{50} values (*Table 3*), it was evident that both **1** and **2** possess promising potential to inhibit the lipoxygenase.

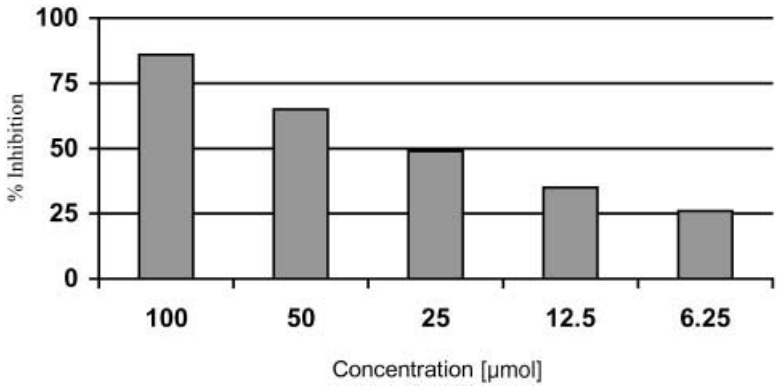


Fig. 3. *Inhibition of lipoxygenase by* **1** *at various concentrations.* For conditions, see *Exper. Part*.

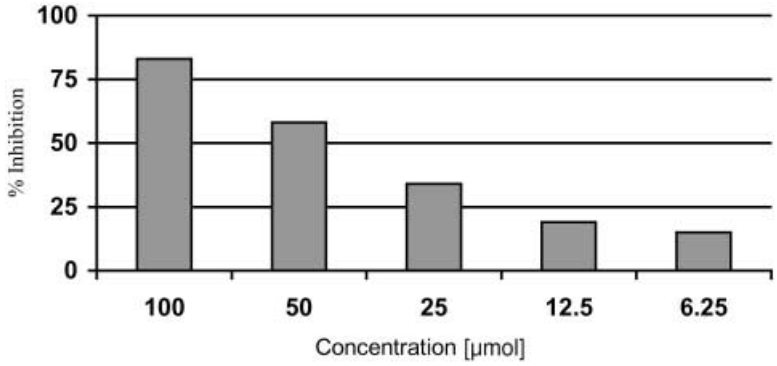


Fig. 4. *Inhibition of lipoxygenase by* **2** *at various concentrations.* For conditions, see *Exper. Part*.

In search of new bioactive substances of plant origin, we have recently studied the chemical constituents of *B. pakistanica* for their antioxidant activity in the $ABTS^{\bullet+}$/HRP/H_2O_2 decoloration assay (*Fig. 5*). The measured IC_{50} values (*Table 4*) showed that only **2** has a significant activity against the $ABTS^{\bullet+}$ radical.

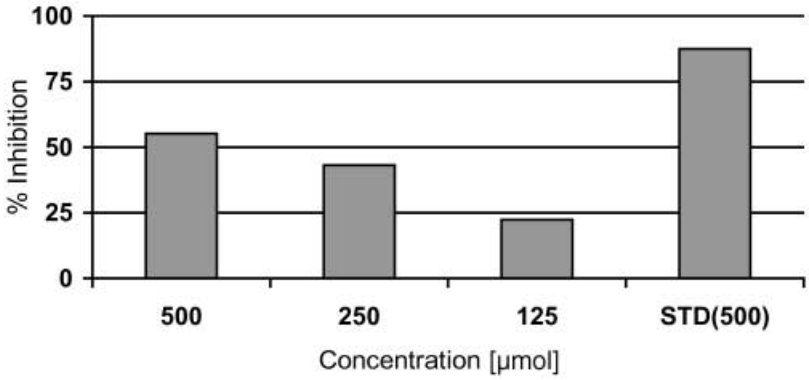


Fig. 5. *Antioxidant activity of* **2** *at various concentrations.* For conditions, see *Exper. Part*.

Table 4. *Antioxidant Activities of the Compounds* **1** *and* **2** *as Compared with the Standard Antioxidant*

	$IC_{50} \pm$ s.e.m.[a]) [µM]
1	–
2	354 ± 15.5
Trolox[b])	87.5 ± 1.0

[a]) Standard error of the mean of three assays. [b]) Standard antioxidant.

Experimental Part

General. Column chromatography (CC): silica gel, 70–230 mesh. Flash Chromatography (FC): silica gel 220–440 mesh. TLC: precoated silica-gel *G-25-UV*$_{254}$ aluminium plates; detection at 254 and 366 nm, and by ceric sulfate reagent. $[\alpha]_D$: *Jasco DIP-360* digital polarimeter. UV Spectra: *Hitachi U-3200* spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: *Jasco A-302* spectrophotometer; $\tilde{\nu}$ in cm^{-1}. NMR: *Bruker* apparatus, at 400 and 500 MHz; δ in ppm rel. to Me_4Si (=0 ppm) as internal standard, *J* in Hz. EI-FAB- and HR-EI-MS: *JMS HX-110* (with data system) and *JMS DA-500* mass spectrometers; *m*/*z* (rel. %).

Plant-Material. *B. pakistanica* (27 kg) was collected from Baluchistan in September 2001 and identified by Prof. *R. B. Tareen*, Department of Botany, University of Baluchistan, Pakistan. A voucher specimen was deposited in the herbarium of the University of Baluchistan.

Extraction and Isolation. Air-dried whole plants of *B. pakistanica* (27 kg) were exhaustively extracted with MeOH at r.t. for two weeks. The MeOH extract was evaporated to a dark greenish mass, which was partitioned initially between hexane/H_2O, AcOEt/H_2O, and then BuOH/H_2O. The AcOEt-soluble fraction was subjected to CC (hexane, hexane/AcOEt, AcOEt, and AcOEt/MeOH, with increasing polarity). The fractions obtained from hexane/AcOEt were combined and subjected to FC (increasing polarity of hexane/AcOEt). The fraction obtained from hexane/AcOEt 6:4, 4:6, 3:7, and 2:8 furnished 7,5′-dimethoxy-3,5,2′-trihydroxyflavone, 4′,5-dihydroxy-3,6,7-trimethoxyflavone, 5,6-dihydroxy-4,7-dimethoxy-2-methylanthracone-9,10-dione, and 1,3,4-trihydroxy-6,7,8-trimethoxy-2-methylanthracene-9,10-dione, respectively. The pure AcOEt fraction was submitted to CC (hexane/AcOEt and AcOEt/MeOH gradients). The fractions obtained with AcOEt/MeOH 9:1 and 9.5:0.5 afforded **1** and **2**, respectively.

Enzyme-Inhibition Assays. The inhibitory activity of the compounds against α-glucosidase from Saccharomyces *sp.* (*Wako Pure Chemical Industries Ltd.*; Wako 076-02841) was measured spectrophotometrically at pH. 6.9 and 37° by using 1 mM 4-nitrophenyl α-D-glucopyranoside (PNP-G) as a substrate and 0.69 units/ml of enzyme, in 50 mM sodium phosphate buffer containing 100 mM NaCl. The 1-deoxynojirimycin (0.425 mM) was used as a positive control. The increment in absorption at 400 nm due to the hydrolysis of PNP-G by α-glucosidase was monitored continuously with the spectrophotometer (*Molecular Devices*, USA) [24].

Activity against lipoxygenase was conveniently measured by slightly modifying the spectrometric method developed by *Tappel* [25]. Lipoxygenase type I-B and linoleic acid were purchased from *Sigma Chemicals* (St. Louis, MO, USA). All other chemicals were of anal. grade. In the assay protocol, 160 µl of 100 mM sodium phosphate buffer (pH 8.0), 10 µl of test-compound soln., and 20 µl of lipoxygenase soln. were mixed and incubated for 10 min at 25°. The reaction was then initiated by the addition of 10 µl of linoleic acid (substrate) soln., with the formation of (9*Z*,11*E*,13*S*)-13-hydroperoxyoctadeca-9,11-dienoate. The change of absorbance at 234 nm was followed for 8 min. Test compounds and the control were dissolved in MeOH. Baicalein (*Aldrich Chem. Co.*) was used as positive control. All the reactions were performed in triplicate on a 96-well microplate in *SpectraMax 340* (*Molecular Devices*, USA). The IC_{50} values were calculated with the EZ-Fit Enzyme kinetics program (*Perrella Scientific Inc.*, Amherst, MA, USA).

Antioxidant Assay. Antioxidant activity was measured by using the $ABTS^{\bullet+}/H_2O_2$/HRP decoloration method. The assay was based on the reduction of a pre-formed $ABTS^{\bullet+}$ by antioxidants. The reaction mixture contained 1 mM of 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] (=2,2′-azinobis[3-ethyl-2,3-dihydrobenzothiazole-6-sulfonic acid]; ABTS), 35 µM H_2O_2, and 6 µM horseradish peroxidase (HRP) in acidified EtOH. In the standard assay (final volume 220 ml/well), the 96-well microtitration plates with flat-bottom wells were supplemented with 20 ml of suitable dilutions of antioxidant compounds. Then 200 ml of the pre-formed $ABTS^{\bullet+}$ was added and the absorbance at 730 nm was read after 6 min [26]. *Trolox* (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid = 3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2*H*-1-benzopyran-2-carboxylic acid) was used as standard antioxidant. The IC_{50} values were calculated with the EZ-Fit Enzyme kinetics program (*Perrella Scientific Inc.*, Amherst, USA).

Pakistolide A (=*4-{[*O-*β*-D-*Glucopyranosyl-(1 → 4)-2*-O-*benzoyl-β*-D-*glucopyranosyl]oxy}benzoic Acid Cyclic Dimeric Ester* = *4-{[*O-*β*-D-*Glucopyranosyl-(1 → 4)*-O-*2*-O-*benzoyl-1*-O-*(1,4-phenylenecarbonyl)-β*-D-*glucopyranosyl-(1 → 6)*-O-*[β*-D-*glucopyranosyl-(1 → 4)]-2*-O-*benzoyl-β*-D-*glucopyranosyl}oxy}benzoic Acid Cyclic Inner Ester*; **1**): Gummy solid (20 mg). $[\alpha]_D^{27} = +30.76$ ($c = 0.026$, MeOH/$CHCl_3$). UV (MeOH): 216 (4.25), 255 (4.09), 296 (3.72). IR (KBr): 3365, 2925, 1652, 1603, 1517, 1220. ^{1}H- and ^{13}C-NMR: *Table 1*. HR-FAB-MS: 1097.3124 ($[M+H]^+$, $C_{52}H_{57}O_{26}^+$; calc. 1097.3138), 549.1595 ($[1/2\ M+H]^+$, $C_{26}H_{29}O_{13}^+$; calc. 549.1608). FAB-MS: 1097 ($[M+H]^+$), 1095 ($[M-H]^+$), 549 ($[1/2\ M+H]^+$), 531 ($[1/2\ M+H-H_2O]^+$), 387 ($[1/2\ M+H-$glucose$]^+$), 282 ($[1/2\ M+H-$benzoylglucose$]^+$). EI-MS: 402 (32, $[1/2\ M+H-$glucose$]^+$), 384 (12), 282 (14), 267 (9), 122 (29), 105 (100), 77 (28).

Pakistolide B (=*4-{[2-*O*-Benzoyl-4-*O*-[(4Z)-1,6-dioxohept-4-enyl]-β-*D*-glucopyranosyl}oxy}benzoic Acid Cyclic Dimeric Ester*=*4-{[*O*-2-*O*-Benzoyl-4-*O*-[(4Z)-1,6-dioxohept-4-enyl]-1-*O*-(1,4-phenylenecarbonyl)-β-*D*-glucopyranosyl-(1*→*6)-2-*O*-benzoyl-4-*O*-[(4Z)-1,6-dioxohept-4-enyl]-β-*D*-glucopyranosyl}oxy}benzoic Acid Inner Ester*; **2**): Gummy solid (18 mg). $[\alpha]_D^{27} = +46.87$ ($c = 0.064$, MeOH/$CHCl_3$). UV (MeOH): 214 (4.21), 254 (4.08), 294 (3.69). IR (KBr): 3361, 2927, 1651, 1602, 1520, 1219. ^{1}H- and ^{13}C-NMR: *Table 2*. HR-FAB-MS: 1021.3119 ($[M+H]^+$, $C_{54}H_{53}O_{20}^+$; calc. 1021.3130), 511.1590 ($[1/2\,M+H]^+$, $C_{27}H_{27}O_{10}^+$; calc. 511.1604). FAB-MS: 1021 ($[M+H]^+$), 511 ($[1/2\,M+H]^+$), 406 ($[1/2\,M+H-$benzoyl$]^+$), 386 ($[1/2\,M+H-$side chain $(C_7H_9O_2)]^+$), 349 ($[1/2\,M+H-$glucose$]^+$), 244 ($[1/2\,M+H-$benzoylglucose$]^+$). EI-MS: 364 (29, $[1/2\,M+H-$glucose$]^+$), 346 (5), 141 (24), 126 (20), 100 (25), 105 (100), 77 (28).

Hydrolysis of **1** *and* **2**. A soln. of **1** or **2** in MeOH (5 ml) and 1N HCl (4 ml) was refluxed for 4 h and then concentrated. The soln. was diluted with H_2O (10 ml) and extracted with AcOEt. The residue recovered from the org. phase was a complex inseparable mixture of products. The aq. phase was evaporated, and D-glucose was identified by the sign of its optical rotation ($[\alpha]_D^{27} = +52.5$) and the GC retention time of its trimethylsilyl ether on comparison with a standard.

The authors express their gratitude to the *Higher Education Commission* for research funding of this project.

REFERENCES

[1] E. Nasir, S. I. Ali, 'The Flora of West Pakistan', Pangraphics Printing Press, Islamabad, 1981, Vol. 140, p. 3.
[2] S. Inoshiri, M. Sasaki, H. Kohda, H. Otsuka, K. Yamasaki, *Phytochemistry* **1987**, *26*, 2811.
[3] N. Sakurai, S. Nagashima, K. Kawai, T. Inoue, *Chem. Pharm. Bull.* **1989**, *37*, 3311.
[4] R. Bekker, E. V. Brandt, D. Ferreira, *Tetrahedron* **1999**, *55*, 10005.
[5] R. Bekker, D. Ferreira, K. J. Swart, E. V. Brandt, *Tetrahedron* **2000**, *56*, 5297.
[6] R. Bekker, R. S. Smit, E. V. Brandt, D. Ferreira, *Phytochemistry* **1996**, *43*, 673.
[7] N. Sakurai, M. Kobayashi, A. Shigihara, T. Inoue, *Chem. Pharm. Bull.* **1992**, *40*, 851.
[8] B. N. Meyer, N. R. Ferrigni, J. E. Putnam, L. B. Jacobsen, D. E. Nichols, J. L. McLaughlin, *Planta Med.* **1982**, *45*, 31.
[9] N. C. Baruah, R. P. Sharma, G. Thyagarajan, W. Herz, S. V. Govindan, *Phytochemistry* **1979**, *18*, 2003.
[10] S. E. Flores, J. Herran, *Tetrahedron* **1958**, *2*, 308.
[11] A. G. Gonzalez, J. B. Barrera, B. B. Davila, E. Valencia, X. A. Dominguez, *Phytochemistry* **1992**, *31*, 255.
[12] R. P. Verma, K. S. Sinha, *Nat. Prod. Lett.* **1994**, *5*, 105.
[13] T. M. Devlin, 'Textbook of Biochemistry with Clinical Correlations', 3rd edn., John Wiley & Sons, New York, 1992, p. 140; K. A. Smith, A. A. Salyers, *J. Bacteriol.* **1991**, *173*, 2962.
[14] H. Matsuura, C. Asakawa, M. Kurimoto, J. Mizutani, *Biosci. Biotechnol. Biochem.* **2002**, *66*, 1576.
[15] T. Matsui, C. Yoshimoto, K. Osajima, T. Oki, Y. Osajima, *Biosci. Biotechnol. Biochem.* **1996**, *60*, 2019.
[16] P. B. Fischer, M. Collin, G. B. Karlsson, W. James, T. D. Butters, S. J. Davis, S. Gordon, R. A. Dwek, F. M. Platt, *J. Virol.* **1995**, *69*, 5791.
[17] H. Kurihara, J. Ando, M. Hatano, *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1241.
[18] J. Adams, B. J. Fitzsimmons, Y. Girard, Y. Leblane, J. F. Evans, J. Rokach, *J. Am. Chem. Soc.* **1985**, *107*, 464; C. R. Pace-Asciak, J. M. Martin, E. J. Corey, W. G. Su, *Biochem. Biophys. Res. Commun.* **1985**, *128*, 942.
[19] D. Steinhilber, *Curr. Med. Chem.* **1999**, *6*, 71.
[20] D. Nie, K. V. Honn, *Cell Mol. Life Sci.* **2002**, *59*, 799.
[21] K. S. Kim, S. Lee, Y. S. Lee, S. H. Jung, Y. Park, K. H. Shin, B. Kim, *J. Ethnopharmacol.* **2003**, *85*, 69.
[22] N. Mukhtar, K. Iqbal, I. Anis, A. Malik, *Phytochemistry* **2002**, *61*, 1005.
[23] M. Inagaki, R. Isobe, Y. Kawano, T. Miyamoto, T. Komori, R. Higuchi, *Eur. J. Org. Chem.* **1998**, 129.
[24] M. S. Ali, M. Jahangir, S. Shahzad-ul-Hussan, M. I. Choudhary, *Phytochemistry* **2002**, *60*, 295.
[25] A. L. Tappel, 'Methods in Enzymology', Academic Press, New York – London, 1962, Vol. 5, p. 539.
[26] A. Cano, M. Acosta, M. B. Arnao, *Redox Report* **2000**, *5*, 365.

Received August 20, 2003