Phosphodiesterase-Inhibiting Glycosides from *Symplocos racemosa*

by **Viqar Uddin Ahmad***, **Muhammad Athar Abbasi**, **Muhammad Zubair**, **Naheed Fatima**, **Umar Farooq**, and **M. Iqbal Choudhary**

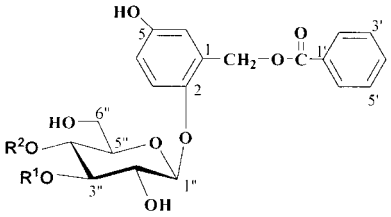
HEJ Research Institute of Chemistry, International Center for Sciences, University of Karachi, Karachi-75270, Pakistan
(fax: (+92)-21-9243190-91; tel: (+92)-21-9243223; e-mail: vuahmad@cyber.net.pk)

Phytochemical investigation of *Symplocos racemosa* resulted in the isolation of two new glycosides, symploracemoside (**1**) and symplomoside (**2**), which are structurally related to the reported benzoylsalireposide (**3**) and salireposide (**4**). The structure elucidation of the isolated compounds was based primarily on 1D- and 2D-NMR analysis, including COSY, NOESY, HMQC, and HMBC correlations. These glycosides showed inhibitory activity against snake-venom phosphodiesterase I.

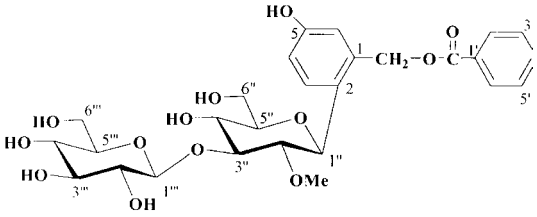
Introduction. – *Symplocos racemosa* ROXB. (LODH) has a wide range of usefulness in indigenous systems of medicine. The astringent bark is recommended in Ayurveda for the treatment of diarrhoea and dysentery, liver complaints, and dropsy. The bark is also used in the treatment of ophthalmia and acts as a tonic and to prevent abortion. In combination with sugar, it is recommended in the treatment of menorrhagia and other uterine disorders [1]. A decoction of it is used as a mouth wash to give firmness to bleeding and spongy gums and taken internally as a snake-bite cure [2]. The glycosides from *Symplocos racemosa* showed inhibitory activity against phosphodiesterase I. Phosphodiesterases are hydrolases that act on diesters of phosphoric acid. Nucleotide pyrophosphatases/phosphodiesterases (NPP) release nucleoside 5′-monophosphates from a variety of nucleotides or nucleotide derivatives [3]. They are widely distributed in mammalian intestinal mucosa, mammalian liver cells, blood serum, snake venom, and various plants [4]. NPPs have been implicated in the regulation of various intra- and extracellular processes including bone and cartilage mineralization, cell differentiation and motility, and signaling by nucleotides and insulin [5][6].

Results and Discussion. – From the AcOEt-soluble fraction of *Symplocos racemosa*, two new compounds, symploracemoside (**1**) and symplomoside (**2**) were isolated which resemble the reported benzoylsalireposide (**3**) [7] and salireposide (**4**) [7] in their structures.

Symploracemoside (**1**) was isolated as a colorless gummy solid. Its FAB-MS showed a M^+ ion peak at m/z 646, corresponding to the molecular formula $C_{35}H_{34}O_{12}$ and indicating 19 degrees of unsaturation. Its UV and IR absorption bands resembled those of benzoylsalireposide (**3**). The acid hydrolysis of **1** provided various products, among which the glycone could be separated and identified as D-glucose on TLC comparison with an authentic sample of this sugar. Its identity was also confirmed by comparing the retention time of the trimethylsilyl ether of the glycone with a standard sample in gas chromatography (GC) and by its optical-rotation sign.



1 R^1 = PhCO, R^2 = 3,4-$(MeO)_2C_6H_3$[1])
3 R^1 = PhCO, R^2 = H[1])
4 R^1 = R^2 = H[1])



2[1])

The EI-MS of **1** exhibited an ion at *m/z* 492 ($[M-(MeO)_2C_6H_3OH]^+$) which further disintegrated into the following characteristic fragments: $[M-C_6H_5CO-(MeO)_2C_6H_3\}+2\,H]^+$ (*m/z* 406, 45%), [*M* – (benzoyl)(dimethoxyphenyl)glucose]$^+$ (*m/z* 244, 13%), $C_6H_3(OH)_2CH_2^+$ (*m/z* 123, 26%), $C_6H_5CO^+$ (*m/z* 105, 100%), and $C_6H_5^+$ (*m/z* 77, 41%). In the ^{1}H-NMR spectrum of **1**, in addition to the signals similar to those of benzoylsalireposide (**3**), peaks appeared at δ 6.88 (*d*, *J* = 2.1 Hz, H–C(2‴)), 6.95 (*d*, *J* = 8.3 Hz, H–C(5‴)), 6.76 (*dd*, *J* = 2.1, 8.3 Hz, H–C(6‴)), 3.87 (*s*, MeO), and 3.82 (*s*, MeO), which are characteristic for a 3,4-dimethoxyphenyl group[1]). These assignments were further confirmed by comparison of the ^{13}C-NMR data with those of the reported 3,4-dimethoxyphenyl β-D-glucopyranoside [8]. The 3,4-dimethoxyphenyl group was placed at C(4″) of the glucose unit of **1** on the basis of NMR data, with an ether linkage to C(4″), since the anomeric C(1″) was involved in a glycosidic bond with the substituted phenolic residue. In the ^{13}C-NMR spectrum, a downfield shift of C(4″) and upfield shifts of C(3″) (although it was acylated) and of C(5″) were observed in **1** as compared to the similar signals of **3** (see *Table*), which confirmed the position of the 3,4-dimethoxyphenyl group at C(4″) of the glucose moiety. In the ^{1}H,^{1}H-COSY plot of **1**, a broad *t* at δ 3.84 was assigned to H–C(2″) on the basis of a cross-peak with the anomeric proton (δ 5.52), the former also showing a cross-peak with a broad *t* at δ 5.21, which thus was assigned to H–C(3″) [7]. This broad *t* at δ 5.21 further gave rise to a cross-peak with a *dd* at δ 4.41, which was assigned to H–C(4″) and shifted to lower field as compared to the corresponding signal of **3** (*Table*), indicating the presence of an ether group at C(4″). These assignments were further confirmed by long-range HMBC data of **1**, the most important being shown in the *Figure*.

Symplomoside (**2**) was isolated as a colorless amorphous solid. Its FAB-MS showed a M^+ ion peak at *m/z* 566, corresponding to the molecular formula $C_{27}H_{34}O_{13}$ and indicating 11 degrees of unsaturation. Its UV and IR spectra resembled those of salireposide (**4**). The acid hydrolysis of **2** provided various products, among which the glycone could be separated. In the hydrolysate separated from the aglycone parts, only D-glucose was detected by TLC, by comparison of the GC retention time of its trimethylsilyl ether with that of a standard, and by its optical-rotation sign, suggesting that one of the sugar moieties present in **2** was linked by a C–C linkage [11].

The EI-MS of **2** exhibited an ion at *m/z* 404 ([*M* – glucose]$^+$), which further disintegrated into the following characteristic fragments: [*M* – glucose-MeOH]$^+$ (*m/z* 372, 5%), $C_6H_5COOH^+$ (*m/z* 122, 28%), $C_6H_5CO^+$ (*m/z* 105, 100%), and $C_6H_5^+$ (*m/z* 77, 42%). In the ^{1}H-NMR spectrum, a set of peaks between δ 7.48 and 8.04 was characteristic of a benzoyl group but the proton signals between δ 6.77–6.98 assigned to H–C(3), H–C(4), and H–C(6)[1]) were slightly shifted as compared to those of benzoylsalireposide (**3**) and salireposide (**4**) (*Table*). The ^{1}H-NMR spectrum additionally showed a *s* at δ 3.85 for a MeO group and a *d* at δ 4.66 (*J* = 7.7 Hz) for an anomeric proton of a β-D-sugar moiety, and the signal of H–C(1″) was shifted upfield to δ 4.45 (*d*, *J* = 9.8 Hz), consistent with the presence of a *C*-(β-D-glucosyl) moiety. In the ^{13}C-NMR spectrum of **2**, the presence of only one acetal anomeric C-atom at δ 99.8 (C(1⁗)) further supported the presence of a *C*-glucosyl

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[1]) Arbitrary numbering (see Formulae **1**–**4**); the ring C-atoms of R^1 are labelled C(1‴) to C(6‴) and those of R^2 C(1⁗) to C(6⁗). For systematic names, see *Exper. Part*.

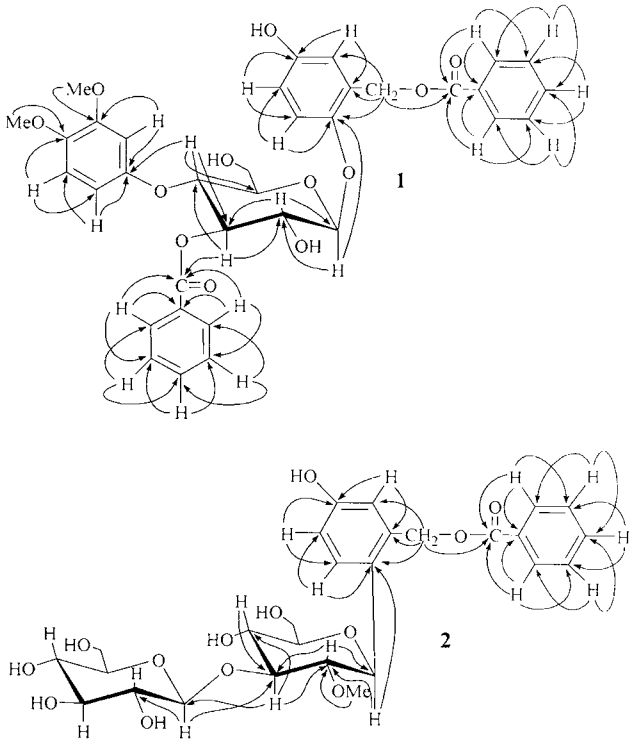

Figure. *Important HMBC correlations of* **1** *and* **2**

moiety. The upfield shifts of C(1″) (δ 80.2) and C(2) (δ 130.1) and down-field shifts of C(1) (δ 144.8) and C(3) (δ 121.9) [9] in comparison with **3** and **4** confirmed the presence of a *C*-glucosyl linkage between C(1″) and C(2); however, the signals of the *C*-glucosyl moiety were different from those of the reported *C*-glycosides [10][11] due to substitution at the *C*-glucosyl moiety and the deshielding effect of the aromatic ring. A downfield shift for C(2″) (δ 82.8) and a cross-peak in the NOESY plot between the MeO (δ 3.85, *s*) and H–C(2″) signal (δ 3.80, *dd*, $J = 7.2$, 7.6 Hz) confirmed the presence of a MeO group at C(2″) [8]. In the ^{1}H,^{1}H-COSY plot, the *dd* at δ 3.80 was assigned to H–C(2″) on the basis of a cross-peak with H–C(1″) (δ 4.45, *d*, $J = 9.8$ Hz), which also caused a cross-peak with the *dd* at δ 3.19 (H–C(3″)). The downfield shift of C(3″) (δ 80.7) in comparison with **4** and a cross-peak in the NOESY between H–C(3″) and the anomeric H–C(1‴) confirmed the linkage between C(3″) and C(1‴). All these assignments were further confirmed by long-range HMBC correlations of **2** (*Figure*).

A very small number of natural inhibitors of phosphodiesterase I has been reported so far. We have already reported the inhibitory activity of benzoylsalireposide (**3**) and salireposide (**4**) against the enzyme phosphodiesterase I. We now also studied the activity of symploracemoside (**1**) and symplomoside (**2**) against the same enzyme. Compound **1** showed moderate inhibitory activity with an IC_{50} of 590 ± 0.0021 μM, while **2** showed weak activity with an IC_{50} of 998 ± 0.0021 μM, as compared to the strong inhibitory potential of **3** (IC_{50} 171 ± 0.002 μM) and the moderate inhibitory activity of **4** (IC_{50} 544 ± 0.0021 μM) relative to cysteine (IC_{50} 748 ± 0.015 μM) and EDTA (IC_{50} 274 ± 0.007 μM). Thus, in terms of structure–activity relationship, the

Table. *NMR Data* (MeOD) *of Compounds* **1**–**4**[a])[1]). δ in ppm, J in Hz

	1[b])		**2**[d])		**3**		**4**	
	δ(H)	δ(C)[c])	δ(H)	δ(C)[c])	δ(H)	δ(C)[c])	δ(H)	δ(C)[c])
C(1)	–	134.0	–	144.8	–	134.1	–	134.3
C(2)	–	149.5	–	130.1	–	149.7	–	149.9
H–C(3)	7.05 (*d*, *J* = 8.7)	119.7	6.86 (*d*, *J* = 8.2)	121.9	7.04 (*d*, *J* = 8.7)	119.6	7.00 (*d*, *J* = 8.8)	119.6
H–C(4)	6.49 (*dd*, *J* = 3.0, 8.7)	115.8	6.77 (*dd*, *J* = 1.8, 8.2)	116.1	6.47 (*dd*, *J* = 3.0, 8.7)	115.4	6.46 (*dd*, *J* = 3.0, 8.3)	115.6
C(5)	–	154.4	–	154.2	–	154.3	–	154.1
H–C(6)	6.79 (*d*, *J* = 3)	116.5	6.98 (*d*, *J* = 1.8)	112.4	6.78 (*d*, *J* = 2.9)	116.0	6.75 (*d*, *J* = 3.0)	116.3
CH_2–C(1)	4.5, 4.72 (2*d*, *J* = 13.2)	64.8	4.52, 4.70 (2*d*, *J* = 12.0)	65.0	4.54, 4.69 (2*d*, *J* = 13.0)	65.1	4.47 (*d*, *J* = 13.1)	65.3
C(1′)	–	131.1	–	131.3	–	131.2	–	131.3
H–C(2′), H–C(6′)	8.01 (*dt*, *J* = 1.5, 7.2)	130.2	8.04 (*dt*, *J* = 1.3, 7.0)	130.6	8.03 (*dt*, *J* = 1.4, 6.1)	130.6	8.02 (*tt*, *J* = 3.0, 8.0)	130.6
H–C(3′), H–C(5′)	7.42 (br. *t*, *J* = 6.9)	129.0	7.48 (br. *t*, *J* = 7.6)	129.6	7.47 (br. *d*, *J* = 7.5)	129.4	7.49 (br. *t*, *J* = 7.4)	129.6
H–C(4′)	7.60 (*m*)	133.8	7.63 (*m*)	134.4	7.61 (*m*)	134.2	7.62 (*m*)	134.0
C(=O)–C(1′)	–	167.4	–	167.9	–	167.7	–	167.8
H–C(1″)	5.52 (*d*, *J* = 6.5)	105.0	4.45 (*d*, *J* = 9.8)	80.2	4.88 (*d*, *J* = 7.8)	104.4	4.78 (*d*, *J* = 2.9)	104.5
H–C(2″)	3.84 (br. *t*, *J* = 7.2)	73.1	3.80 (*dd*, *J* = 7.2, 7.6)	82.8	3.86 (br. *t*, *J* = 7.5)	73.4	3.40–3.51 (*m*)	75.1
H–C(3″)	5.21 (*t*, *J* = 9.4)	76.9	3.19 (*dd*, *J* = 7.8, 7.9)	80.7	5.33 (*t*, *J* = 9.3)	79.3	3.40–3.51 (*m*)	78.0
H–C(4″)	4.41 (*dd*, *J* = 6.3, 6.4)	84.6	3.89 (br. *t*, *J* = 7.4)	69.9	3.88 (br. *t*, *J* = 7.5)	70.3	3.40–3.51 (*m*)	72.0
H–C(5″)	3.67 (*ddd* *J* = 1.5, 7.2, 9.5)	74.8	3.82*m*	79.8	3.78 (*ddd*, *J* = 1.6, 7.7, 9.6)	75.4	3.70 (*dd*, *J* = 2.2, 7.3)	75.6
CH_2(6″)	4.46, 4.76 (2*dd*, *J* = 7.1, 11.3)	60.5	4.49, 4.73 (2*dd*, *J* = 5.8, 11.9)	62.6	4.48, 4.72 (2*dd*, *J* = 7.2, 11.6)	60.7	4.42 (*dd*, *J* = 7.3, 11.7)	61.0
C(1‴)	–	131.2			–	131.6		
H–C(2‴), H–C(6‴)	8.10 (*dt*, *J* = 1.3, 7.4)	130.4			8.10 (*dt*, *J* = 1.4, 7.0)	130.7		
H–C(3‴), H–C(5‴)	7.48 (br. *t*, *J* = 6.8)	129.2			7.51 (br. *d*, *J* = 7.7)	129.6		
H–C(4‴)	7.62 (*m*)	133.9			7.61 (*m*)	134.4		
C(=O)–C(1‴)	–	167.5			–	167.8		

[a]) All spectra were recorded at 500 (^{1}H) and 125 MHz (^{13}C); assignments were supported by 2D-NMR COSY, HMQC, and HMBC experiments. [b]) δ (H) of R^2 of **1**: 6.88 (*d*, *J* = 2.1, H–C(2⁗)); 6.95 (*d*, *J* = 8.3, H–C(5⁗)); 6.76 (*dd*, *J* = 2.1, 8.3, H–C(6⁗)); 3.87 (*s*, MeO–C(3⁗)); 3.82 (*s*, MeO–C(4⁗)). δ(C) of R^2 of **1**: 148.2 C(1⁗)); 105.8 (C(2⁗)); 155.0 (C(3⁗)); 151.3 (C(4⁗)); 110.6 (C(5⁗)); 114.9 (C(6⁗)); 56.2 (*Me*O–C(3⁗)); 56.8 (*Me*O–C(4⁗)). [c]) ^{13}C-NMR multiplicities were determined by DEPT 135°. [d]) Additional δ(H) of **2**: 3.85 (*s*, MeO–C(2″)); 4.66 (*d*, *J* = 7.7, H–C(1‴)); 3.61 (br. *t*, *J* = 7.9, H–C(2‴)); 3.64 (br. *t*, *J* = 8.2, H–C(3‴)); 3.52 (br. *t*, *J* = 7.5, H–C(4‴)); 3.82 (*m*, H–C(5‴)); 4.49, 4.73 (2*dd*, *J* = 5.8, 11.9, CH_2(6‴)). Additional δ(C) of **2**: 56.5 (*Me*O–C(2″)); 99.8 (C(1‴)); 74.9 (C(2‴)); 77.2 (C(3‴)); 72.0 (C(4‴)); 75.1 (C(5‴)); 62.0 (C(6‴)).

presence of a 3,4-dimethoxyphenyl group in **1** at C(4″) resulted in a decrease of biological activity against the enzyme phosphodiesterase I as compared to that of **3**. Similarly, the presence of a *C*-glucosyl linkage in **2** and also the presence of a MeO group at C(2″) and a glucose moiety at C(3″) resulted in a further decrease of biological activity against phosphodiesterase I as compared to that of **4**.

Experimental Part

General. Column chromatography (CC): silica-gel, 70–230 mesh. Flash chromatography (FC): silica gel, 230–400 mesh. TLC: pre-coated silica-gel *G-25-UV*$_{254}$ plates; detection at 254 nm and by ceric sulfate reagent. Optical rotations: *Jasco DIP-360* digital polarimeter. UV Spectra: *Hitachi UV-3200* spectrometer; λ_{max} (log ε) in nm. IR Spectra: *Jasco 320-A* spectrophotometer; in cm^{-1}. ^1H- and ^{13}C-NMR, COSY, HMQC, and HMBC Spectra: *Bruker* spectrometers operating at 500 and 400 MHz; chemical shifts δ in ppm and coupling constants J in Hz. EI- and FAB-MS: *JMS-HX-110* with a data system; in *m/z* (rel. int.).

Plant Material. The plant *Symplocos racemosa* (Symplocaceae) was collected from Abbottabad, Pakistan, in January 2001, and identified by Dr. *Manzoor Ahmed* (taxonomist), Department of Botany, Post-Graduate College, Abbottabad, Pakistan. A voucher specimen (no. 6453) has been deposited at the herbarium of the Department of Botany, Post-Graduate College, Abbottabad, Pakistan.

Extraction and Purification. The shade-dried ground bark (30 kg) was exhaustively extracted with MeOH at r.t. and the extract evaporated. The residue (818 g) was extracted with hexane, $CHCl_3$, AcOEt, and BuOH. The AcOEt extract (106.2 g) was subjected to CC (silica gel, hexane with $CHCl_3$ gradient ($\rightarrow$ 100%), followed ley MeOH): *Fractions 1–10*. *Fr. 8* was submitted to FC (silica gel 230–400 mesh, MeOH/$CHCl_3$ 5:95): *Fr. 8.1* and *8.2*. *Fr. 8.2* was subjected to prep. TLC (MeOH/acetone/$CHCl_3$ 0.5:35:64.5): **1**. *Fr. 10* was subjected to CC (MeOH/$CHCl_3$ 8:92): *Fr. 10.1–10.3*. *Fr. 10.1* was subjected to prep. TLC (MeOH/acetone/$CHCl_3$ 0.5:42:57.5) to purify compound **2**.

Acid Hydrolysis of **1** *and* **2**. A soln. of compound **1** or **2** (6 mg) in MeOH (5 ml) containing 1N HCl (4 ml) was refluxed for 4 h, evaporated, and diluted with H_2O (8 ml). The mixture was extracted with AcOEt and the org. extract evaporated to give an inseparable mixture of products. The aq. phase was evaporated and the residue analyzed by TLC: presence of D-glucose when compared with an authentic sample. The identity of the sugar was also confirmed by comparing the GC t_R of the Me_3Si ether of the glycone with a standard sample α-D-anomer, t_R 4.1 min; β-D-anomer, t_R 7.8 min) and by the sign of optical rotation ($[\alpha]_D^{20} = +52$) ($c = 1$, H_2O).

Symploracemoside (= *2-[(Benzoyloxy)methyl]-4-hydroxyphenyl 4-O-(3,4-Dimethoxyphenyl)-β-D-glucopyranoside 3-Benzoate*; **1**). Colorless gummy solid (14.2 mg). $[\alpha]_D^{23} = -7.14$ ($c = 0.056$, MeOH). UV (MeOH): 282.1 (3.12), 228.8 (3.17), 208.2 (3.80), 193.8 (2.7). IR (KBr): 3379 (OH); 2925 (C–H); 1722 (C=O, ester); 1602, 1498, 1454 (C=C, Ar); 1273, 1213 (C–O–C); 1118–1031 (C–O); 756, 713, 671. ^1H- and ^{13}C-NMR: *Table*. FAB-MS (pos.-ion mode): 647 ($C_{35}H_{35}O_{12}^+$; $[M+H]^+$). FAB-MS (neg.-ion mode): 645 ($[M-H]^-$). EI-MS: 492 (16.9, $[M-CH_3O)_2C_6H_3OH]^+$), 406 (45, $[M-C_6H_5CO-(CH_3O)_2C_6H_3+2\,H]^+$), 244 (13, [*M* – (benzoyl)(dimethoxyphenyl)glucose]$^+$), 137 (49, $(MeO)_2C_6H_3^+$), 123 (26, $C_6H_3(OH)_2CH_2^+$), 105 (100, $C_6H_5CO^+$), 77 (41, $C_6H_5^+$).

Symplomoside (=*1-C-[2-[(Benzoyloxy)methyl]-4-hydroxyphenyl] O-β-D-Glucopyranosyl-(1→3)-(1-deoxy-2-O-methyl-β-D-glucopyranoside*; **2**). Colorless amorphous solid (15.3 mg). $[\alpha]_D^{23} = +7.5$ ($c = 0.06$, MeOH). UV (MeOH): 280.2 (2.68), 230 (3.50), 206.2 (3.64), 196 (2.1). IR (KBr): 3373 (OH); 2922 (C–H); 1722 (C=O, ester); 1602, 1516, 1454 (C=C, Ar); 1277 (C–O–C); 1119–1030 (C–O); 715,669. ^1H- and ^{13}C-NMR: *Table*. FAB-MS (pos.-ion mode): 567 ($C_{27}H_{35}O_{13}^+$, $[M+H]^+$). FAB-MS (neg.-ion mode): 565 ($[M-H]^-$). EI-MS: 404 (14, [*M* – glucose]$^+$), 372 (12, [*M* – glucose – MeOH]$^+$), 122 (28, $C_6H_5CO_2H^+$), 105 (100, $C_6H_5CO^+$), 77 (42, $C_6H_5^+$).

Enzyme Inhibition Assay. Activity against snake-venom phosphodiesterase I (*Sigma P 4631*; EC 3.1.4.1) was assayed by using the reported method [12] with the following modifications: 33 mM *Tris*·HCl buffer pH 8.8, 30 mM $Mg(OAc)_2$ with 0.000742 U/well final concentration by using a microtiter-plate assay and 0.33 mM bis-(4-nitrophenyl) phosphate (*Sigma N-3002*) as substrate. Cysteine and EDTA [3] [13–15] from *Merck* were used as positive controls ($IC_{50} = 748 \pm 0.015$ μM and 274 ± 0.007 μM, resp.). After 30 min pre-incubation of the enzyme with the test samples, enzyme activity was monitored spectrophotometrically at 37° on a microtiter-plate reader (*SpectraMax*, *Molecular Devices*) by following the rate (change in *OD*/min) of release of 4-nitrophenol from bis(4-nitrophenyl) phosphate at 410 nm. All assays were conducted in triplicate.

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