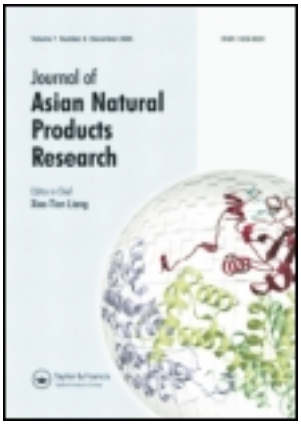

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Sorbicins A and B, new urease and serine protease inhibitory triterpenes from *Sorbus cashmiriana*

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Two new lupene-type triterpenes, sorbicins A (**1**) and B (**2**), have been isolated from the chloroform-soluble fraction of the MeOH extract from the whole plant of *Sorbus cashmiriana*, and their structures were elucidated by spectroscopic techniques including 2D NMR. Both compounds displayed urease and α-chymotrypsin inhibitory potential.

Keywords: *Sorbus cashmiriana*; sorbicins A and B; urease; α-chymotrypsin

1. Introduction

The genus *Sorbus* belongs to the family Rosaceae and comprises 200 species of trees and shrubs that are commonly found in Asia, Africa, and South America. The genus is represented in Pakistan by seven species. One of these is *Sorbus cashmiriana* Hedlung, Monog, which is a tree found in Kashmir and the western Himalayas. A tea made from its bark is used to treat nausea. Bark preparation is also used to treat heart diseases. Berries are used to cure scurvy [1–5]. The literature survey revealed that triterpenes have previously been reported from this plant [6,7]. The presence of bioactive compounds could be inferred by the strong toxicity of MeOH extract of *S. cashmiriana* in a brine shrimp lethality test [8]. Pharmacological screening of the extract revealed strong inhibitory activity against the enzymes urease and α-chymotrypsin. On further fractionation, major inhibitory activity was detected in the chloroform-soluble fraction, and bioassay-directed fractionation resulted in the isolation of two new lupene-type triterpenes named sorbicins A (**1**) and B (**2**) (Figure 1).

Urease (E.C. 3.5.1.5) has been shown to be an important virulent determinant in the pathogenesis of many clinical conditions that are detrimental to human and animal health as well as in agriculture. Urease is directly involved in the formation of infection stones and contributes to the pathogenesis of urolithiasis, pyelonephritis, hepatic encephalopathy, hepatic coma, and urinary catheter encrustation [9,10]. Therefore, strategies based on urease inhibition are now considered as the first line of treatment for infections caused by urease-producing bacteria.

The physiological roles of serin protease inhibitors have been clearly established. It has been proposed that they are

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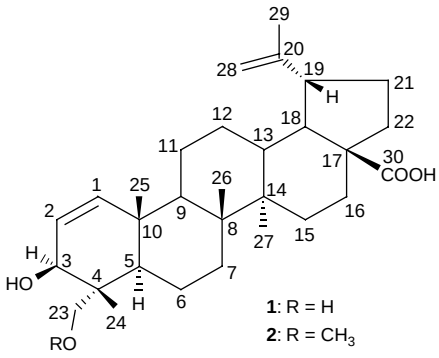

Figure 1. Structures of compounds **1** and 2.

part of plants' natural defense system against insect predation and function by inhibiting insect proteases [11,12]. Hence, these inhibitors have gained attention as possible sources of engineered resistance against pests and pathogens for transgenic plants expressing heterologous inhibitors [12]. Serin proteases such as chymotrypsin and trypsin are involved in the destruction of certain fibrous proteins [13].

Chronic infection by hepatitis C virus can lead to progressive liver injury, cirrhosis, and liver cancer. A chymotrypsin-like serin protease known as NS3 protease, which has very similar active site as chymotrypsin is thought to be essential for viral replication and has become target for anti-HIV drugs [14].

In this study, we have described the urease and protease inhibitory activities of the new triterpenoids **1** and **2**, respectively.

2. Results and discussion

The MeOH extract of the whole plant of *S. cashmiriana* was processed as described in the Experimental section to afford two new triterpenes named sorbicins A (**1**) and B (**2**). Both **1** and **2** gave positive Salkowski and Lieberman–Burchard tests for triterpenes and evolved brisk effervescence with dilute $NaHCO_3$ solution indicating the presence of free carboxylic acid.

Sorbicin A (**1**) was obtained as an amorphous solid. The IR spectrum showed the presence of hydroxyl groups (3400 cm^{-1}), a carbonyl group (1708 cm^{-1}), and double bond functionalities (1640–1660 cm^{-1}). The HR-EI-MS showed a molecular ion peak at *m/z* 470.3390 consistent with the molecular formula $C_{30}H_{46}O_4$ possessing eight degrees of unsaturation. The ^{13}C NMR spectrum showed 30 signals comprising five methyl, ten methylene, eight methine groups, and seven quaternary carbons (Table 1). The carbonyl carbon of the carboxylic group resonated at δ 178.9, while the olefinic carbon atoms resonated at δ 150.2, 140.5, 128.8, and 109.7. The oxymethine carbon was observed at δ 75.2, while oxymethylene carbon resonated at δ 68.1. The ^1H NMR spectrum displayed signals of five tertiary methyl groups at δ 0.78 (s, 3H), 1.00 (s, 3H), 1.08 (s, 3H), 1.13 (s, 3H), and 1.72 (s, 3H), an isopropenyl group [δ 1.72 (s, 3H), 4.84 (br s, 1H), and 4.76 (br s, 1H)], a double doublet at δ 3.85 (dd, $J = 2.5$, 1.4 Hz, 1H) for the oxymethine proton and doublets at δ 3.47 (d, $J = 11.1$ Hz, 1H) and 3.22 (d, $J = 11.1$ Hz, 1H) for the hydroxymethylene group. The above data indicated that sorbicin A (**1**) is a lupene-type triterpene with two of the methyl groups oxidized to form hydroxymethylene and carboxylic moieties. The EI-MS showed a characteristic fragment at *m/z* 425 resulting from the loss of 45 $[M-COOH]^+$, revealing the presence of free carboxylic group at the angular C-17 position. It was further authenticated by HMBC spectrum (Figure 2). The protons at δ 2.11 and δ 1.28 showed ^3J correlations with C-28 (δ 178.9). The partial structure C—CH = CH—HC (OH)—C was indicated by signals at δ 5.42 (dd, $J = 11.5$, 2.5 Hz, 1H), 5.34 (dd, $J = 11.5$, 1.4 Hz, 1H), and 3.85 (dd, $J = 2.5$, 1.4 Hz, 1H). This partial structure can exist only in ring A either as lupa-1,20(29)-dien-3-ol or lupa-2,20(29)-dien-1-ol, and it was proved to be the

Table 1. ^{13}C and ^{1}H NMR spectral data of **1** and **2** (δ in ppm, J in Hz, in $CDCl_3$).

	1		**2**	
No.	δ_C	δ_H	δ_C	δ_H
1	140.5	5.34 (dd, 11.5, 1.4)	140.5	5.33 (dd, 11.5, 1.4)
2	128.8	5.42 (dd, 11.5, 2.5)	128.8	5.42 (dd, 11.5, 2.5)
3	75.2	3.85 (dd, 1.4, 2.5)	75.9	3.84 (dd, 1.4, 2.5)
4	40.1	–	39.8	–
5	55.0	1.34 (m)	55.2	1.32 (m)
6	19.6	1.00 (m)	19.6	1.01 (m)
7	32.1	0.95 (m)	32.1	0.96 (m)
8	39.0	–	39.0	–
9	39.6	1.85 (m)	39.6	1.83 (m)
10	35.0	–	35.0	–
11	23.4	1.66 (m)	23.5	1.67 (m)
12	26.6	0.90 (m)	26.6	0.90 (m)
13	40.7	1.19 (m)	40.7	1.18 (m)
14	42.3	–	41.6	–
15	26.0	1.59 (m)	26.1	1.59 (m)
16	30.5	1.27 (m)	30.7	1.27 (m)
17	48.1	–	48.0	–
18	43.0	2.11 (d, 4.9)	43.2	2.11 (d, 4.9)
19	49.2	3.12 (dd, 4.9, 10.7)	49.4	3.14 (d, 4.9, 10.7)
20	150.2	–	150.1	–
21	29.7	1.20 (m)	29.7	1.21 (m)
22	34.1	1.28 (m)	34.1	1.27 (m)
23	68.1	3.47 (d, 11.1), 3.22 (d, 11.1)	71.4	3.47 (d, 11.1), 3.22 (d, 11.1)
24	20.5	0.78 (s)	20.8	0.79 (s)
25	15.9	1.13 (s)	15.0	1.11 (s)
26	21.0	1.08 (s)	20.9	1.06 (s)
27	15.8	1.00 (s)	15.6	1.00 (s)
28	178.9	–	178.9	–
29	109.7	4.84 (s), 4.76 (s)	109.7	4.85 (s), 4.75 (s)
30	19.3	1.72 (s)	19.3	1.72 (s)
23-OCH_3			56.2	3.43 (s)

former by similarity of NMR chemical shifts of ring A with those of glochidol [15] and further confirmed through HMBC correlations. The proton at δ 5.34 showed ^{2}J correlations with C-2 (δ 128.8) and C-10 (δ 35.0) as well as ^{3}J correlations with C-3 (δ 75.2), C-5 (δ 55.0), C-9 (δ 39.6), and C-25 (δ 15.9). The proton at δ 5.42 showed ^{2}J correlations with C-1 (δ 140.5) and C-3 (δ 75.2) as well as ^{3}J correlations with C-10 (δ 35.0) and C-4 (δ 40.1). Similarly, the proton at δ 3.85 showed ^{2}J correlations with C-2 (δ 128.8), C-4 (δ 40.1) as well as ^{3}J correlations with C-1 (δ 140.5), C-5 (δ 55.0), C-23 (δ 68.1), and C-24 (δ 20.5).

The stereochemistry of the hydroxyl group at C-3 was assigned β and equatorial based on the presence of strong interactions

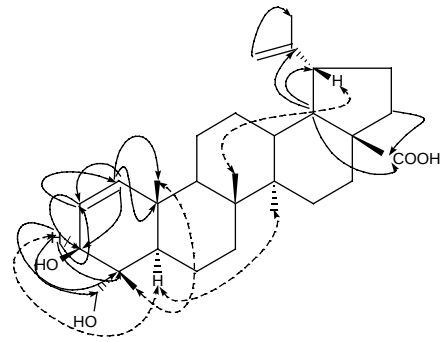

Figure 2. Key HMBC (→) and NOESY (←– –→) correlations for compound **1**.

between H-3, H-5, and H_3-27 in NOESY spectrum. The hydroxymethyl group was assigned to C-4 on the basis of HMBC correlations described in Table 1. The β-orientation of C-24 could be assigned by comparing its ^{13}C NMR signal with those of related compounds [16–18] and further confirmed by NOESY interactions of the protons of H-24 with H-25. The signal at δ 3.12 (dd, $J = 4.9$, 10.7 Hz, 1H) could be assigned to H-19 in β and axial configuration. On the basis of these evidences, sorbicin A (**1**) was assigned the structure 3β,23-dihydroxylupa-1,20(29)-dien-28-oic acid (Figure 1).

Sorbicin B (**2**) was also obtained as an amorphous powder. The IR spectrum was very similar to that of **1**. The molecular formula was deduced as $C_{31}H_{48}O_4$ by HR-EI-MS showing an $[M]^+$ peak at *m/z* 484.3559. The ^1H and ^{13}C NMR spectra were also similar to those of **1** except the presence of additional signals due to a methoxy group (δ 3.43 and δ 56.2). It could be assigned to C-23 due to the downfield shift of C-23 signal compared to **1** and also on the basis of HMBC experiment showing ^3J correlation of the methoxyl at δ 3.43 with C-23 (δ 71.4). The rest of the HMBC and NOESY correlations were similar to those of **1**, allowing us to retain the same relative configuration. The structure of sorbicin B (**2**) could thus be assigned as 3β-hydroxy-23-methoxylupa-1,20(29)-dien-28-oic acid (Figure 1).

Compounds **1** and **2** displayed inhibitory potential with IC_{50} values of 85.2 ± 0.28 and 17.8 ± 0.12 μM against Jack bean urease, respectively. The standard inhibitor (thiourea) had an IC_{50} value of 21.6 ± 0.18 μM. Thus, the methylation of hydroxyl group at C-23 has a marked pharmacophoric effect on the urease inhibitory activity. Both compounds **1** and **2** inhibited α-chymotrypsin enzyme in a concentration-dependent manner with IC_{50} values of 23.2 ± 0.09 and 22.7 ± 0.12 μM, respectively, whereas the positive control, chymostatin, had an IC_{50} value of 7.2 ± 0.26 μM. Therefore, both the isolated compounds **1** and **2** have significant potential to bind and inhibit α-chymotrypsin enzyme.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured with a JASCO DIP-360 digital polarimeter ($l = 10$ cm). IR spectra were measured on JASCO-320-A spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Bruker AM-400 spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C NMR) in $CDCl_3$ with tetramethylsilane as internal standard. Chemical shifts δ are shown in ppm relative to tetramethylsilane. The HR-EI-MS were recorded on a Jeol JMS-HX mass spectrometer in *m/z* (%). Thin layer chromatographies (TLC) were carried out on pre-coated silica gel F_{254} plates (E. Merck, Darmstadt, Germany, 0.25 and 0.50 mm thickness, respectively) detected at 254 nm and by spraying with ceric sulfate reagent. Silica gel 230–400 mesh (E. Merck) was used for column chromatography (CC). All chemicals, Jack bean urease (E.C. 3.5.1.5), and α-chymotrypsin were purchased from Sigma Chemical Company (St. Louis, MO, USA).

3.2 Plant material

The whole plant of *S. cashmiriana* was collected in June 2008 from Kashmir (Pakistan) and identified by Dr Surraiya Khatoon, Plant Taxanomist, Department of Botany, University of Karachi, Karachi, Pakistan, where a voucher specimen (No. KUH 73/67 760) has been deposited.

3.3 Extraction and isolation

The freshly collected whole plant material (20 kg) was cut into small pieces and extracted with MeOH (3 × 30 l). The combined MeOH extracts were evaporated under reduced pressure to yield a residue

(900 g), which was divided into *n*-hexane (70 g), $CHCl_3$ (95 g), EtOAc (180 g), *n*-BuOH (65 g), and H_2O (48 g) soluble fractions. The $CHCl_3$-soluble fraction was subjected to CC over SiO_2 and eluted with mixtures of EtOAc–MeOH in increasing order of polarity. The fractions that eluted with EtOAc–MeOH (9.5:0.5) were a binary mixture of compounds. These were combined and again subjected to CC over SiO_2 eluting with mixtures of EtOAc–MeOH in increasing order of polarity. The fractions that eluted with EtOAc–MeOH (9.9:0.1) were combined and subjected to preparative TLC with EtOAc–MeOH (9.8:0.2) as development solvent to afford sorbicin B (**2**) (17 mg). The fractions that eluted with EtOAc–MeOH (9.7:0.3) were combined and subjected to preparative TLC (EtOAc–MeOH [9.8:0.2]) to obtain sorbicin A (**1**) (19 mg).

3.3.1 *Sorbicin A (**1**)*

Amorphous solid: $[\alpha]_D^{20}$ − 39.0 (*c* 1.0, CH_3OH); IR (KBr) υ_{max} cm^{-1}: 3400, 1708, 1640–1660; For ^{13}C and ^{1}H NMR spectral data, see Table 1. HR-EI-MS *m/z*: 470.3390 $[M]^+$ (calcd for $C_{30}H_{46}O_4$, 470.3396).

3.3.2 *Sorbicin A (**2**)*

Amorphous powder: $[\alpha]_D^{20}$ − 57.0 (*c* 1.0, CH_3OH); IR (KBr) υ_{max} cm^{-1}: 3400, 1708, 1640–1660. For ^{13}C and ^{1}H NMR spectral data, see Table 1. HR-EI-MS *m/z*: 484.3559 $[M]^+$ (calcd for $C_{31}H_{48}O_4$, 484.3552).

3.4 In vitro *urease inhibitory assay*

Reaction mixtures containing 25 μl of enzyme (Jack bean urease) solution and 55 μl of buffers containing 100 mM urea were incubated with 5 μl of test compounds at 30°C for 15 min in 96-well plates. Urease activity was determined by measuring ammonia production using the indophenol method of Weatherburn [19]. Briefly, 45 μl each of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 μl of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added to each well. The increasing absorbance at 630 nm was measured after 50 min, using a microplate reader Spectromax, 384 plus (Molecular Device, Sunnyvale, CA, USA). All reactions were performed in triplicate in a final volume of 200 μl. The results (change in absorbance per min) were processed using Soft Max Pro software (Molecular Device). All the assays were performed at pH 8.2 (0.01M $K_2HPO_4{\cdot}3H_2O$, 1 mM ethylenediaminetetraacetic acid and 0.01 M LiCl). Percentage inhibition was calculated from the formula 100 − (Optical density test well/Optical density control) × 100. Thiourea was used as the standard inhibitor of urease.

3.5 In vitro *chymotrypsin inhibitory assay*

The chymotrypsin inhibitory activity was performed by the method of Cannel et al. [20]. Chymotrypsin (9 units/ml of 50 mM Tris–HCl buffer, pH 7.6) was pre-

Table 2. *In vitro* quantitative inhibition of urease and α-chymotrypsin by **1** and **2**.

Drug	Urease inhibition IC_{50} ± SEM (μM)[a]	α-Chymotrypsin inhibition IC_{50} ± SEM (μM)[a]
1	85.2 ± 0.28	23.2 ± 0.09
2	17.8 ± 0.12	22.7 ± 0.12
Thiourea[b]	21.6 ± 0.18	–
Chymostatin[b]	–	7.2 ± 0.26

[a] SEM, standard error of the mean ($n = 3-5$)
[b] Positive control.

incubated with various concentrations of test compounds for 20 min at 25°C, respectively, and then 100 µl of substrate solution (*N*-succinyl-phenylanin-*p*-nitroanilide, 1 mg/ml of 50 mM Tris–HCl buffer pH 7.6) was added to start the enzyme reaction (Table 2). The absorbance of released *p*-nitroaniline was continuously monitored at 410 nm until a significant color change was achieved. The final DMSO concentration in the reaction mixture was 7%. The IC_{50} value was determined by monitoring the effect of various concentrations of test compounds in the assays on the inhibition values. The IC_{50} values were then calculated using the FZ-FIT enzyme kinetics program (Perella Scientific, Inc., Amherst, MA, USA).

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