Chymotrypsin Inhibitory Triterpenoids from *Silybum marianum*

Ejaz AHMED,[a] Abdul MALIK,*[,a] Sadia FERHEEN,[b] Nighat AFZA,[b] AZHAR-UL-HAQ,[a] Muhammad Arif LODHI,[a] and Muhammad Iqbal CHOUDHARY[a]

[a] *International Center for Chemical Sciences, H.E.J. Research Institute of Chemistry, University of Karachi; Karachi-75270, Pakistan: and* [b] *Pharmaceutical Research Center, PCSIR Laboratories, Karachi Complex; Karachi-75280, Pakistan.*
Received May 16, 2005; accepted August 16, 2005

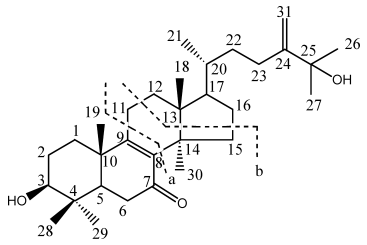
Marianine, the new lanostane triterpene (1) and marianosides A (2) and B (3) have been isolated from the whole plant of *Silybum marianum*. Their structures were elucidated on the basis of extensive analysis of their one dimensional and two dimensional nuclear magnetic resonance (1D, 2D-NMR) spectral data. All inhibited chymotrypsin in a concentration-dependent manner.

Key words *Silybum marianum*; marianine; marianoside A; marianoside B; chymotrypsin inhibition

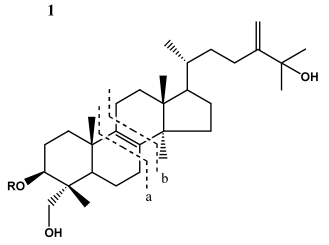
Silybum marianum GAERTH is an erect stout biennial, herb belonging to the family Compositae which comprises about 1000 genera and 20000 species. *S. marianum* is common in Kashmir at an altitude of 1800—4000 m and is cultivated for ornaments in gardens. The plant is used in the treatment of hepatitis, cirrhosis, regeneration from liver diseases, and liver poisoning.[1)] A decoction of this plant is also used by the local physicians for the treatment of a variety of viral diseases.[1)] A literature survey revealed that twenty compounds have so far been reported from *S. marianum*. The major constituents are, however, flavonoids, flavanolignans, diterpenes and flavanoglycosides.[2,3)] The ethnopharmacological and chemotaxonomic importance of the genus *Silybum* prompted us to investigate the chemical constituents of *S. marianum*. A methanolic extract of this plant showed strong toxicity in the brine shrimp lethality test, revealing the possible presence of bioactive compounds. Further pharmacological screening revealed significant inhibitory activity against the enzyme chymotrypsin. The inhibitory activity was most pronounced in the chloroform-soluble fraction. This prompted us to carry out bioassay-guided isolation of bioactive compounds from this fraction. As a result of these studies we have isolated marianine, a new lanostane-type triterpene (**1**) and marianosides A (**2**) and B (**3**), two new triterpenoidal glucosides, respectively. All three showed significant chymotrypsin inhibiting activity. This is the first report of the natural occurrence of lanostane-type triterpenes in the genus *Silybum*.

Results and Discussion

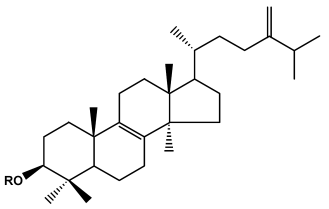
Marianine (**1**) was isolated from the chloroform fraction of *S. marianum* as colorless crystals. It gave color reactions of triterpene and the molecular formula was determined to be $C_{31}H_{50}O_3$ by $[M]^+$ peak in high-resolution (HR) EI-MS at *m*/*z* 470.3735 (Calcd 470.3759), indicating seven degrees of unsaturation. The strong IR absorptions at 3415, 1635 and 1670 indicated the presence of hydroxyl, olefinic, and carbonyl functionalities. Absorption at 249 nm in the UV spectrum suggested the presence of a conjugated carbonyl system in (**1**). The mass spectrum gave fragment ions 'a' at *m*/*z* 206 and 'b' at *m*/*z* 261, which are characteristic of lanostane triterpenes carrying $\Delta^{8(9)}$-7- one moiety.[4—8)] Further peaks at *m*/*z* 188 and 243 resulted from the loss of a water molecule from both these fragments. It also indicated the presence of one hydroxyl group in ring A or B. The carbinylic proton at δ 3.32 showed ^1H–^1H correlation spectroscopy (COSY) with two other protons limiting its location to either C-1 or C-3. However, it could be assigned to the usual C-3 position on the basis of heteronuclear multiple-bond connectivity (HMBC) as illustrated in Fig. 2. The larger coupling constant of this proton allowed us to assign β and equatorial configurations to the hydroxyl group which was substantiated by nuclear overhaueser effect (NOE) between H-3 and H-28. The loss of the side chain resulted in a fragment ion at *m*/*z* 329, showing the presence of one hydroxyl and an olefinic moiety in the side chain. Since both methyl groups at C-25 were observed downfield as singlets, the hydroxyl group could be referred to C-25 and confirmed by the HMBC correlations (Fig. 2). The methylene group could be assigned to C-24



1



2 R = β-D- glucosyl
2a R = H



3 R = β-D- glucosyl
3a R = H

Fig. 1. Structures of Compounds **1**—**3**

* To whom correspondence should be addressed. e-mail: abdul.malik@iccs.edu

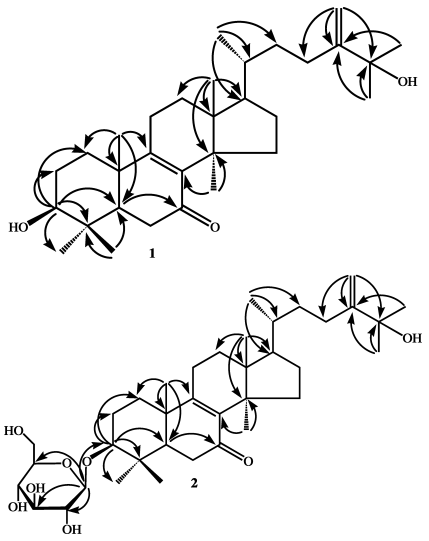


Fig. 2. Important HMBC Correlations in Marianine (**1**) and Marianoside A (**2**)

with the help of HMBC correlations and further confirmed by a comparison of proton chemical shifts with those of related compounds reported in the literature.[8,9] The ^{13}C-NMR spectrum [broad band (B.B) & distortionless enhancement by polarization transfer (DEPT)] showed the presence of 31-carbon signals including methyls, methylene, methane and quaternary carbons which could be assigned on the basis of heteronuclear multiple-quantum coherence (HMQC) (Table 1). Thus, marianine (**1**) could be assigned as 24-methylene-7-oxo-lanosta-8-(9)-ene-3β,25-diol.

Marianoside (**2**) was obtained as a white amorphous powder and was positive in the Lieberman Burchard test for a triterpene. The positive mode HR-FAB-MS showed $[M+H]^+$ ion at *m/z* 619.4545 (Calcd 619.4573) corresponding to the molecular formula $C_{37}H_{62}O_7$ with seven degrees of unsaturation. Strong IR bands were observed at ν_{max} 3445—3420 (OH group), 3030, 1640 (C=C), and 1420 cm^{-1} (gem-dimethyl). The ^{1}H-NMR spectrum of **2** showed the characteristic signal for an anomeric proton as a doublet at δ 4.21 (J=7.7 Hz). The larger coupling constant inferred the β-configuration of the anomeric position. The ^{1}H-NMR spectrum further showed the signal of six tertiary methyl groups at δ 0.72, 0.97, 1.10, 1.05, 1.30, and 1.32 and a secondary methyl at 0.88 (3H, d, J=7.5 Hz). The oxymethine protons of the sugar moiety were observed from δ 3.40 to 3.05 and the oxymethylene protons showed signals at δ 3.61 (1H, dd, J=11.1, 4.8 Hz) and 3.58 (1H, dd, J=11.1, 5.8 Hz). The presence of an additional hydroxymethyl was evident by downfield signals at δ 4.20 (1H, d, J=11.2 Hz) and 3.66 (1H, d, J=11.2 Hz). The attachment of the sugar moiety shifted the carbinylic proton resonance to δ 3.99.

Acid hydrolysis provided the sugar and the aglycon, respectively. The former could be identified as D-glucose through comparative thin layer chromatography (co-TLC) with an authentic sample of D-glucose, and sign of its optical rotation ($[\alpha]_D$ +52.6°). The aglycon was obtained as colorless crystals from methanol and showed the molecular ion in HR-EI-MS at *m/z* 472.3950 corresponding to the molecular formula $C_{31}H_{52}O_3$. The loss of $C_9H_{17}O$ (side-chain) gave a peak at *m/z* 331, revealing that the side-chain is similar to **1**.

Table 1. ^{1}H-NMR Data for Compounds **1**—**3**

Position	**1**	**2**	**3**
1α	1.86 dt (13.1, 3.3)	1.88 m	1.75 dd (11.5, 6.5)
1β	2.06 m	1.95 m	1.23 m
2	1.75 m	1.80 m	1.84 m
3α	3.29 dd (4.6, 11.6)	3.99 dd (10.5, 5.1)	3.82 dd (11.1, 4.9)
5α	1.67 m	1.38 m	1.37 m
6α	2.49 dd (3.9, 15.8)	2.10 m	2.10 m
6β	2.38 dd (12.4, 15.8)	1.63 m	1.63 m
7	—	2.20 (dd, 11.5, 8.1) 2.10 m	2.03 m
11α	2.37 m	2.11 m	2.06 m
11β	2.24 ddd (4.2, 4.2, 2.0)		
12	1.80 m	1.43 m	1.19 m
15α	1.56 m	1.52 m	1.95 m
15β	2.13 m	1.88 m	2.10 m
16α	1.33 m	1.43 m	1.72 m
16β	1.93 m	1.56 m	1.95 m
17	1.43 m	1.12 m	1.68 m
18	0.72 s	0.68 s	0.66 s
19	1.05 s	1.33 s	1.35 s
20	1.43 m	1.44 m	1.32 m
21	0.88 d (6.0)	0.85 d (6.2)	0.86 d (6.5)
22	1.11 m	1.14 m	1.11 m
23	1.85 m 2.10 m	1.88 m 2.06 m	1.85 m 2.10 m
25	—	—	2.22 m
26	1.00 s	1.00 s	0.99 d (6.9)
27	0.99 s	0.99 s	1.01 d (6.9)
28	0.97 s	3.61 d (11.2) 4.20 d (11.2)	0.97 s
29	0.85 s	1.03 s	0.95 s
30	0.95 s	0.94 s	0.84 s
31	4.62 s	4.64 br s	4.64 s
1′		4.21 d (9.69)	4.30 d (7.8)
2′		3.05 m	3.06 m
3′		3.40 m	3.40 m
4′		3.11 m	3.14 m
5′		3.07 m	3.06 m
6′		3.58 dd (11.1, 4.8) 3.61 dd (11.1, 5.8)	3.88 dd (11.5, 4.5) 3.80 dd (11.5, 5.0)

The coupling constants are given in parentheses in Hz.

This was further substantiated by proton chemical shifts of the side chain which showed complete agreement to those of **1**. The fragmentation pattern was similar by comparing the data with the compounds reported in the literature,[10—15] with ions 'a' and 'b' appearing at *m/z* 184 and 264, respectively. This indicated that both the OH groups were present in rings A or B. The downfield oxymethine proton at δ 3.99 showed ^{1}H–^{1}H COSY correlations with two other protons limiting its presence to C-1 or C-3 or C-7. It could be assigned to C-3 on biogenetic grounds and further confirmed by the HMBC correlations. The large coupling constant for H-3 allowed us to assign β and equatorial orientation to the hydroxyl group. Since the signals of C-10 methyl showed the same chemical shift as that in **1**, the hydroxymethyl group could be assigned to C-4 and subsequently confirmed by the HMBC correlations. In the ^{13}C-NMR spectra (BB and DEPT) 37 signals

were observed including seven methyl, thirteen methylene, nine methine, and eight quaternary carbons. The chemical shifts of H_2-28 [δ 3.61 (d, J=11.2 Hz), δ 4.20 (d, J=11.2 Hz)] and H_3-29 (δ 1.03) suggested an equatorial orientation of the hydroxymethyl group. It has been noted previously that, in triterpenoids with C-4 attached hydroxymethyl groups, the 4β methyl group resonates about to 10 ppm further upfield than the corresponding 4β-oriented methyl group.[16—19] Thus, the aglycon **2a** could be assigned 24-methylene-lanoesta-8(9)-ene-25,28-diol. The ^{13}C-NMR spectrum, ^{1}H–^{1}H COSY correlations and HMQC were in complete agreement to the assigned structure. The structure of the corresponding glucoside **2** could thus be elucidated as the 3-*O*-β-D-glucoside of **2a**.

Marianoside B (**3**) was isolated as an amorphous solid. The molecular formula was determined by positive mode HR-FAB-MS showing $[M+H]^+$ ion at m/z 603.4652 corresponding to the molecular formula $C_{37}H_{63}O_6$. The IR spectrum showed absorption bands for hydroxyl groups at 3421—3380 cm^{-1}, for a C=C group at 1634 cm^{-1}, and for terminal methylenes at 1650 and 887 cm^{-1}. Acid hydrolysis provided the sugar moiety which could be identified as D-glucose through Co-TLC with an authentic sample and sign of its optical rotation ($[\alpha]_D$ +52.9°). The larger coupling constant for the anomeric proton in the ^{1}H-NMR spectrum at δ 4.30 (J=9.4 Hz) allowed us to assign the β configuration to the anomeric position. The aglycon (**3a**) crystallized from methanol, melted at 159—161 °C and showed $[\alpha]_D^{25}$ +63.0°. The physical and spectral data were in complete agreement with those reported in the literature for eburicol (**3a**).[20] Thus, marianoside B (**3**) could be assigned as 24-methylene-lanosta-8(9)-ene 3-*O*-β-D-glucopyranoside.

Serine protease inhibitors have been proposed to be part of the plant's natural defense system against insect predation, and function by inhibiting insect proteinases.[21—24] Hence, these inhibitors have gained attention as possible sources of engineered resistance against pests and pathogens for transgenic plants expressing heterologous inhibitors.[25] Chronic infection by hepatitis C virus can lead to progressive liver injury, cirrhosis, and liver cancer. A chymotrypsin like serine protease known as NS3 protease thought to be essential for viral replication has become a target for anti-HCV drugs.[26,27] The search for new and effective inhibitors of serine proteases is an urgent need in drug development, and new chymotrypsin inhibitors appears to be a promising approach. Compounds **1**—**3** showed significant inhibition against chymotrypsin.

Experimental

General Experimental Procedure UV and IR spectra were recorded on Hitachi-UV-3200 and Jasco-320-A spectrometers, respectively. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AM-400 spectrometer with tetramethylsilane (TMS) as an external standard. The 2D-NMR spectra were recorded on a Bruker AMX 500 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 Finnigan MAT 12 or MAT 312 spectrometers and ions are given in m/z (%). TLC was performed with precoated silica gel G-25-UV$_{254}$ plates and detection was done at 254 nm, and by spraying with ceric sulphate in 10% H_2SO_4. Silica gel (E. Merck, 230—400 mesh) was used for column chromatography. Melting points were determined on a Gallenkemp apparatus and are uncorrected. For enzyme inhibition assay, all chemicals and chymotrypsin were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Plant Material The whole plant of *Silybum marianum* GAERTH was collected from Margalla Hills near Islamabad, in March 2004 and identified by Dr. Rubina Ashraf, a plant taxonomist at the National Agriculture and Research Center Islamabad (NARC), Pakistan, where a voucher specimen has been deposited.

Extraction and Purification The air dried whole plant (25 kg) was exhaustively extracted with methanol (50 l×3) at room temperature. The extract was evaporated to yield a residue (750 g), which was partitioned between *n*-hexane (150 gm), chloroform (80 g), ethyl acetate (55 g), *n*-butanol (68 g) and water (38 g). The chloroform-soluble fraction was subjected to column chromatography over silica gel with *n*-hexane–$CHCl_3$, $CHCl_3$, $CHCl_3$–MeOH, in increasing order of polarity. The fractions obtained from *n*-hexane : chloroform (1.5 : 8.5) were combined and rechromatographed over silica gel with *n*-hexane–$CHCl_3$ (1.2 : 8.8) to afford marianine **1** (19 mg). The fractions obtained from $CHCl_3$: MeOH in (8.0 : 2.0) were combined and rechromatographed over silica gel with $CHCl_3$: MeOH in increasing order of polarity. The fractions obtained from $CHCl_3$: MeOH (8.5 : 1.5) were subjected to preparative TLC ($CHCl_3$: MeOH, 7.8 : 2.2) to afford the pure marianoside A **2** (22 mg) and marianoside B **3** (28 mg).

Marianine (**1**): Colorless crystals; mp 160—161 °C. ^{1}H- and ^{13}C-NMR ($CDCl_3$, 500 MHz and 125 MHz, respectively) see Tables 1 and 2. IR (KBr) ν_{max} cm^{-1}: 3410, 3300, 1655, 1670, 1450, 1375, 1034, 870, 725. UV λ_{max} ($CHCl_3$) nm (log ε): 249 (3.75), 202 (3.58). EI-MS m/z (rel. int.) 470 (20), 452 (28), 455 (100), 437 (28), 380 (40), 365 (36), 329 (75) 288 (60), 261 (70), 243 (74), 221 (55), 239 (42), 206 (62), 188 (66), 178 (48), 160 (25), 99 (29), 85 (18). HR-EI-MS m/z 470.3735 (Calcd for $C_{31}H_{50}O_3$, 470.3760). $[\alpha]_D^{20}$ +73° (c=1.0, $CHCl_3$).

Marianoside A (**2**): Amorphous solid; mp 271—273 °C. ^{1}H- and ^{13}C-NMR (C_5D_5N, 500 MHz and 125 MHz, respectively) see Tables 1 and 2. IR

Table 2. ^{13}C-NMR Spectral Data of the Compound **1** (δ ppm, in $CDCl_3$), and **2**, **3** (δ ppm, in C_5D_5N)

Position	1	2	3
1	35.8	30.7	30.9
2	27.8	23.5	23.8
3	78.9	78.1	79.9
4	38.7	36.8	38.8
5	49.1	44.4	49.5
6	37.9	37.3	37.0
7	202.7	26.2	25.9
8	152.0	134.1	134.3
9	149.8	134.9	135.0
10	39.7	37.7	37.9
11	21.1	21.0	21.1
12	30.7	30.9	30.2
13	44.3	44.7	44.2
14	50.0	50.1	50.0
15	31.4	31.1	31.3
16	28.5	28.4	28.6
17	50.5	50.9	50.7
18	16.0	15.7	16.1
19	20.8	20.9	20.8
20	36.0	36.3	36.1
21	18.5	18.5	18.5
22	33.3	34.8	33.0
23	31.3	31.3	38.0
24	157.8	156.8	156.9
25	72.6	72.8	33.8
26	30.0	30.3	21.9
27	30.0	30.01	22.0
28	28.0	65.3	28.2
29	16.0	13.2	14.5
30	17.2	18.1	17.1
31	106.2	106.1	106.1
1′		100.7	101.0
2′		70.0	70.0
3′		76.7	76.3
4′		73.4	73.0
5′		76.6	76.0
6′		61.0	61.3

Table 3. *In Vitro* Quantitative Inhibition of Chymotrypsin

Compound	IC_{50}±S.E.M.[a] (μM)
1	9.4±0.02
2	22.6±0.1
3	28.2±0.8
Chymostatin[b]	7.01±0.1

a) Standard mean error (S.E.M.) of three experimental determinations. *b*) Positive control used in assays.

(KBr) ν_{max} cm^{-1}: 3445, 3420, 3380, 1640, 1430, 1210, 1040, 810, 708. EI-MS *m*/*z* (rel. int.) 472 $[M]^+$ (33), 454 (25), 457 (100), 436 (31), 421 (40), 387 (55), 331 (28), 288 (66), 264 (88), 208 (62), 184 (44), 182 (60), 141 (44), 85 (18). HR-FAB-MS *m*/*z* 619.4555 (Calcd for $C_{37}H_{63}O_7$ $[M+H]^+$, 619.4573). $[\alpha]_D^{25}$ −28° (*c*=1.0, MeOH).

Marianoside B (**3**): Amorphous solid; mp 240—242 °C. ^{1}H- and ^{13}C-NMR (C_5D_5N, 500 MHz and 125 MHz, respectively) see Tables 1 and 2. IR (KBr) ν_{max} cm^{-1}: 3421, 3380, 1634, 1650, 1445, 1425, 1360, 1240, 1005, 930, 887. HR-FAB-MS *m*/*z* 603. 4655 (Calcd for $C_{37}H_{63}O_6$ $[M+H]^+$, 603.4624). $[\alpha]_D^{25}$ −45.5° (*c*=0.1, MeOH).

Chymotrypsin Inhibition Assay Chymotrypsin inhibitory activity of compounds **1**—**3** was assayed (Table 3) by the method of Cannell *et al*.[28] Chymotrypsin (9 units/ml in 50 μM Tris–HCl buffer pH 7.6, Sigma Chemical Co., U.S.A.) was preincubated with compounds **1**—**3** for 20 min at 25 °C. 100 μl of substrate solution (*N*-succinyl-phenylalanine-*p*-nitroanilide, 1 mg/ml of 50 mM Tris–HCl buffer, pH 7.6) was added to start the enzyme reaction. 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%.

Estimation of IC_{50} Values The concentrations of test compounds **1**—**3** that inhibited the hydrolysis of substrates (chymotrypsin) by 50% (IC_{50}) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The IC_{50} values were then calculated using with the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, U.S.A.).

Acid Hydrolysis of Compounds 2 and 3 A solution of **2** or **3** (8 mg) in methanol (4 ml) and 1 M HCl (4 ml) was refluxed for 4 h. The solution was concentrated under reduced pressure and diluted with H_2O (4.5 ml). It was extracted with ethyl acetate and the sugar in the aqueous phase was identified as D-glucose by the sign of its optical rotation ($[\alpha]_D$ +52.6 from **2** and +52.9 from **3**) and co-TLC with an authentic sample of D-glucose using a solvent system consisting of *n*-BuOH–EtOAc–HOAc–H_2O (12 : 2 : 2 : 2). TLC was run three times in the same direction and spots were visualized with aniline phthalate reagent.

In the case of **3**, the known aglycon, eburicol **3a**, was obtained which was crystallized from MeOH, mp 159 °C. The melting point and spectral data were in complete agreement with those reported in the literature.[20]

References

1) Majda H., Mojca S., Zeljko K., Davorin B., *Food Chem.*, **74**, 355—364 (2001).
2) Kalonga M., *Z. Naturforsch. B*, **36**, 262—265 (1981).
3) Lee D. Y.-W., Yanze L., *J. Nat. Prod.*, **66**, 1171—1174 (2003).
4) Wagne H., Seligmann O., Hörhammer L., Sonnenbichler M. S., *Tetrahedron Lett.*, 1895—1899 (1971).
5) Yoshikawa K., Nishimura N., Bando S., Arihara S., Eiko M., Katayama S., *J. Nat. Prod.*, **65**, 548—552 (2002).
6) Wang L. Y., Wang N. L., Yao X. S., Miyata S., Kitanaka S., *J. Nat. Prod.*, **66**, 630—633 (2003).
7) Tanaka R., Kasubuchi K., Kita S., Tokuda H., Nishino H., Matsunaga S., *Phytochemistry*, **63**, 99—103 (2000).
8) Tanaka R., Kasubuchi K., Kita S., Matsunaga S., *Phytochemistry*, **51**, 457—463 (1999).
9) Lue Y. P., Mu Q., Zheng H. L., Li C. M., *Phytochemistry*, **49**, 2053—2056 (1998).
10) Hasan C. M., Shahnaz S., Muhammad I., *J. Nat. Prod.*, **50**, 762—763 (1987).
11) Tokuyama C. T., Nishizawa M., Shiro M., Tokuda H., Hayashiji Y., *Phytochemistry*, **29**, 923—928 (1990).
12) Knight S. A., *Org. Mag. Res.*, **6**, 603—607 (1974).
13) Knight S. A., *Tetrahedron Lett.*, **14**, 83—86 (1973).
14) Khuong-Huu F., Sangare M., Chari V. M., Devys B. M., Barbier M., Lukacs G., *Tetrahedron Lett.*, **22**, 1787—1790 (1975).
15) Li G. M., Segi M., Nakajima T., *Tetrahedron Lett.*, **33**, 3515—3518 (1972).
16) Seo S., Tomita Y., Tori K., *Tetrahedron Lett.*, **16**, 7—10 (1975).
17) Fullas F., Wani M. C., Wall M. E., Tucker J. C., Becher C. W., Kinghorn A. D., *Phytochemistry*, **43**, 1303—1318 (1996).
18) Nakatani M., Miyazaki Y., Iwashita T., Naoki H., Hase T., *Phytochemistry*, **28**, 1479—1482 (1989).
19) Amimoto K., Yoshikawa K., Arihara S., *Phytochemistry*, **33**, 1475—1480 (1993).
20) Willian W. E., George V. L., *J. Org. Chem.*, **31**, 3434—3435 (1996).
21) Amtul Z., Rahman A., Siddiqui R. A., Choudhary M. I., *Curr. Med. Chem.*, **9**, 1323—1327 (2002).
22) Boulter D., Gatehouse A. M., Hilder V., *Biotechnol. Adv.*, **7**, 489—497 (1989).
23) Ryan C. A., *Annu. Rev. Phytopathol.*, **28**, 425—429 (1990).
24) Masoud S. A., Johnson L. B., White F. F., Reeck G. R., *Plant Mol. Biol.*, **21**, 655—659 (1993).
25) Hilder V. A., Gatehouse A. M. R., Sheerman S. E., Barker R. F., Boulter D., *Nature* (London), **330**, 160—162 (1987).
26) Starkey P. M., *Acta Biol. Med. Germ.*, **36**, 1549—1554 (1977).
27) Patrick A. K., Potts K. E., *Clin. Microb. Rev.*, **11**, 614—617 (1998).
28) Cannell R. J. P., Kellam S. J., Owsianka A. M., Walker J. M., *Planta Med.*, **54**, 10—14 (1988).