Molluscicidal Activity and New Flavonoids from Egyptian *Iris germanica* L. (var. *alba*)

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Dedicated to the late Dr. Amer H. Ahmed

The molluscicidal activity of leaf and rhizome extracts of *Iris germanica* L. (var. *alba*) against *Biomphalaria alexandrina* snails was evaluated and the rhizome extracts were found to be the most potent. Activity-guided fractionation revealed that the chloroform extract showed the highest molluscicidal activity (LC_{90} = 1.26 mg/l) among the tested extracts of the rhizomes. Fraction B prepared from the chloroform extract was the most potent molluscicide (LC_{90} = 0.96 mg/l) in addition, it showed a significant heart rate reduction in the snail after a 6- to 24-h exposure period. It also displayed a significant level of cercaricidal potential in a time-concentration relationship pattern. Chromatographic fractionation and purification of fraction B resulted in the isolation of two novel compounds: 5,2′-dihydroxy-3-methoxy-6,7-methylenedioxyflavone and 5,7,2′-trihydroxy-6-methoxyflavanone. Their structures were established by one- and two-dimensional NMR methods and mass spectrometry.

Key words: Iris germanica L. (var. *alba*), Molluscicidal Activity, Flavonoids

Introduction

Iris species possess vast medicinal purposes and are used in the treatment of cancer, inflammation, bacterial and viral infections (Hanawa *et al.*, 1991). The compounds isolated from these species were reported to have piscicidal, antineoplastic, antioxidant, anti-tumor, antiplasmodial, and antituberculosis properties (Hideyuki *et al.*, 1995; Miyake *et al.*, 1997; Bonfils *et al.*, 2001). *Iris germanica* L. is cultivated as an ornamental plant and widely distributed all over the world.

Schistosomiasis is an endemic disease caused by helminthes belonging to the genus *Schistosoma.* This disease affects more than 200 million people and places with more than 600 million inhabitants are at risk of infection in more than 70 countries in the tropics (WHO, 1994). In view of its prevalence and morbidity this disease is a serious public health problem in many countries. The search for molluscicidal compounds derived from local plants in endemic areas infested by Schistosomes could help the developing countries to control the vector snails with easy and cheap procedures. Previous work on *Iris pseudacorus* L., an ornamental plant cultivated in Egypt, proved its molluscicidal activity (Sary *et al.*, 2004). In continuation of our search for molluscicidal agents from natural source, *Iris germanica* L. was selected as a rich source of active constituents (Hideyuki *et al.*, 1995; Miyake *et al.*, 1997; Bonfils *et al.*, 2001).

Activity-guided fractionation was followed during the course of extraction and separation. Successive extracts of *Iris germanica* L. rhizomes were evaluated for their molluscicidal activity against *Biomphalaria alexandrina* adult snails. The chloroform extract showed the highest molluscicidal activity (LC_{90} = 1.26 mg/l). Fractionation of this extract using CC on a LH-20 column gave a fraction, rich in flavonoid compounds, which showed potent molluscicidal activity (LC_{90} = 0.96 mg/l). This fraction has a significantly high level of cercaricidal activity. The median lethal dose (0.5 mg/l) of this fraction showed a significant heart rate reduction in snails after a 6- to 24-h exposure period.

Chromatographic purification of this fraction resulted in the isolation of two novel compounds

5,2′-dihydroxy-3-methoxy-6,7-methylenedioxyflavone (**1**) and 5,7,2′-trihydroxy-6-methoxyflavanone (**4**) together with known isoflavones, irilin A (**2**) and irilin B (**3**) (Fig. 1). Structures were determined by high-resolution positive-ion mass spectrometry and NMR spectroscopy. The complete assignment of proton and carbon signals was achieved by 2D-NMR experiments: HSQC, HMBC, DQF-COSY and NOESY. In view of these findings, this study was undertaken to evaluate the molluscicidal activity of *Iris germanica* L. against *Biomphalaria alexandrina* and cercariae (free living stages). Also the study was extended to determine the active fraction and to identify its main components

Materials and Methods

Plant material and extraction

Leaves and rhizomes of *Iris germanica* L. (var. *alba*) were collected during the flowering stage (April 2004) from the Faculty of Agriculture at Moshtohor, Banha University, Banha, Egypt. The plant materials were kindly identified by Professor Abd El Salaam M. Al-Nowiahi, Professor of Plant Taxonomy, Faculty of Science, Ain Shams University, Abbassia, Cairo, Egypt. A voucher specimen (IRg-2004) has been deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University. The fresh rhizomes (750 g) of *Iris germanica* L. were cleaned under running tap water, cut into small pieces and extracted successively with *n*-hexane, $CHCl_3$ and finally with 70% ethanol at room temperature. Each extract was concentrated under vacuum at a temperature not exceeding 45 °C to give 13 g, 9 g and 38 g samples, respectively. 100 g of fresh leaves and rhizomes were extracted separately with 70% ethanol and dried under vacuum for studying the preliminary molluscicidal activity of leaves and rhizomes.

Activity-guided fractionation of $CHCl_3$ extract

7.2 g of the active molluscicidal $CHCl_3$ extract were chromatographed over an LH-20 column and eluted with MeOH/$CHCl_3$ (1:1 v/v) to give 2 fractions (A and B). Fraction A gave an oily residue (1.5 g) showing negative tests for flavonoids, while fraction B yielded a yellow semisolid material (5.3 g) after concentration under reduced pressure, giving positive tests for flavonoids (ferric chloride and Shinoda tests). The two fractions were further subjected to a molluscicidal activity test.

*Isolation of compounds **1–4***

Fraction B was the active fraction among the extracts and the fractions of *Iris germanica* L. So, 4 g were subjected to LH-20 CC and eluted with MeOH/$CHCl_3$ (1:1 v/v) to afford 8 fractions. Fraction 7 gave a crystalline deposit that was purified on an LH–20 column by eluting with $CHCl_3$/MeOH (4:1 v/v) to give 4 mg of compound **1**. Fraction 5 showed 2 spots using TLC with petroleum ether/acetone (4:1 v/v) as solvent; this fraction was subjected to preparative TLC eluted with petroleum ether/acetone (4:1 v/v). Each band was eluted with methanol to give 5 mg of compound **2** and 4 mg of compound **3**. Fraction 4 showed a major spot impure with minor materials, which was purified over a silica gel column by eluting with *n*-hexane with increasing the amount of acetone to give 10 mg of pure yellow crystalline compound **4**.

Determination of molluscicidal activity

Biomphalaria alexandrina (8–10 mm in size), hosts of *Schistosoma mansoni*, were collected from irrigation canals in Giza Governorate and kept in laboratory conditions for a period not less than 3 weeks before being used in toxicity experiments. The procedure applied for screening tests on adult snails is based on the professional testing technique recommended by the WHO (1965). Stock solutions were prepared by dissolving 1 g of a dried alcoholic extract in the minimal amount of ethanol, then made up to 1000 mg/l concentration by adding a suitable volume of distilled water. Series of dilutions that permit the computation of LC_{50} and LC_{90} values were prepared. Each concentration of the plant extracts was tested 5 times using 10 snails in 500 ml dechlorinated water per experiment. A similar number of control snails was maintained in normal dechlorinated water under the same conditions. The snails were considered to be dead when they were retracted in their shells and discolored. Data analysis aimed to determine the LC_{50} and LC_{90} values was carried out using the computer software of Finney (1971).

Determination of cercaricidal activity

The effect of fraction B on the mortality of *S. mansoni* cercariae was assessed. *B. alexandrina* snails that had been experimentally infected with

miracidia of *S. mansoni* were allowed to shed cercariae which were then pooled into a glass beaker. Fifty cercariae were counted as previously described (Ramzy *et al.*, 1991) and exposed in a Petri dish to different concentrations of fraction B. The same number of cercariae was placed in Petri dishes containing dechlorinated tap water as a control group. Each dilution, as well as control group, was tested in triplicate. The cercariae were viewed under a stereomicroscope and considered to be dead when they stopped all movement. LC_{100} values were determined after 5, 30 and 60 min exposure periods.

Determination of snail heart rate

Twenty five adult *B. alexandrina* snails of equal size (8 mm) were maintained in dechlorinated tap water, at room temperature ranged from 25–27 °C. Snails were placed individually under a dissection microscope and the time required for 10 ventricular contractions is recorded as the control or pre-exposure time. The snails were exposed to the median lethal dose (0.5 mg/l) of fraction B in a glass jar filled with 2 l of the extract dilution, and the heart rate of each snail was recorded at 2, 6 and 24 h exposure periods. The recorded time required for 10 ventricular contractions in the control and experimental snails was converted to beats/min (Cheng and Sullivan, 1973). Student's *t*-test was applied to determine significant differences between treated and control snails. All comparisons having a probability < 0.05 were considered to be significant.

General experimental procedures

Column chromatography (CC) was performed on silica gel (70–230 mesh), purchased from E. Merck (Darmstadt, Germany), and Sephadex LH-20 (25–100 mesh, Pharmacia). Thin layer chromatography (TLC) was performed on pre-coated silica G 60 F_{254} sheets, 0.25 mm (E. Merck), and KC_{18} RP plates (10 × 20 cm, 200 μm, Whatman). Pre-coated preparative silica gel F_{254} plates with 2 mm thickness were used for isolation of compounds **2** and **3**. The HPLC-DAD system consisted of a Merck-Hitachi L-6200A pump connected to a Perkin-Elmer LC-235 UV-diode array detector and a Perkin-Elmer GP-100 graphics printer. The LSIMS (Cs) mass spectra were recorded on a VG ZABSpec mass spectrometer (VG Analytical, Manchester, UK) in the positive-ion mode using 3-nitrobenzyl alcohol as matrix and poly(ethylene glycol) as a reference compound for accurate mass measurements. Accurate mass measurements were obtained at a resolving power of ca. 5000 by ESA voltage scanning by LSIMS or EI^+ ionization (only for irilins A and B). NMR spectra were acquired using Bruker Avance 500 and 600 spectrometers (equipped with BBI-5mm-Zgrad-ATM and BBO-5mm-Zgrad probes) operating at 500.13 and 600.13 MHz for ^1H and 125.77 and 150.90 MHz for ^{13}C NMR spectroscopy, respectively. Spectra were recorded at 25 °C using CD_3OD as solvent with a non-spinning sample in 5-mm NMR-tubes. Spectra were processed by a PC with the Windows XP operating system and XWin-NMR software. Proton and carbon NMR spectra were referenced internally to a TMS signal using the value 0.00 ppm. In addition to basic ^1H and ^{13}C NMR spectra, also two-dimensional techniques DQF-COSY, NOESY, HSQC and HMBC with gradient selection were utilized. All spectra were measured by the pulse programs originally installed by Bruker.

Results and Discussion

Molluscicidal activity

A preliminary molluscicidal test for total crude ethanol extract of the rhizomes and leaves of *Iris germanica* L. against *Biomphalaria alexandrina* adult snails showed that the total alcohol extract of the rhizomes was more effective [with LC_{50} 1.3 mg/l (1.2–1.4 mg/l)] than the total alcohol extract of leaves [with LC_{50} 49.6 mg/l (49.1–50.1 mg/l)] after a 24-h exposure, respectively. The data of successive extracts of rhizomes are presented in Table I. According to Mott (1987), the alcoholic or lipophilic plant extract is considered as an active molluscicide if it has LC_{90} values equal or less than 20 mg/l after 24 h. Among the successive extracts

Table I. Molluscicidal activity of successive extracts of *Iris germanica* L. rhizomes against *B. alexandrina* after a 24-h exposure period.

Extract	Activity [mg/l] LC_{50} (95% CI)[a]	LC_{90} (95% CI)	Slope[b]
n-Hexane	47.1 (44.0–50.4)	81.9 (71.7–93.4)	1.54
Chloroform	0.64 (0.59–0.70)	1.3 (1.1–1.5)	1.69
Ethanol	24.4 (23.0–26.2)	42.8 (37.2–49.2)	1.54

[a] CI, confidence interval.
[b] Mortality (probit) vs. log concentration (Finney, 1971).

Table II. Molluscicidal activity of chloroform fractions of *Iris germanica* L. rhizomes against *B. alexandrina* after a 24-h exposure period.

Fraction	Activity [mg/l] LC_{50} (95% CI)[a]	LC_{90} (95% CI)	Slope[b]
A	36.4 (33.2–39.8)	74.9 (63.0–89.0)	1.75
B	0.49 (0.45–0.53)	1.0 (0.8–1.1)	1.7

[a] CI, confidence interval.
[b] Mortality (probit) vs. log concentration (Finney, 1971).

examined, only the chloroform extract showed an LC_{90} less than 20 mg/l (LC_{90} = 1.3 mg/l), while the ethanol and *n*-hexane extracts showed low molluscicidal activities. Activity-guided fractionation was followed to evaluate the toxicity of the chloroform extract. The data of molluscicidal activity of the two fractions (A and B) prepared from the chloroform extract are presented in Table II. Fraction B was found to contain flavonoid compounds and it showed a significantly potent molluscicidal activity (LC_{90} of 1.0 mg/l). This finding is in agreement with the data reported for molluscicidal activity of lipophilic flavonoids of *Millettia thonningii* (Perrett and Whitfield, 1995).

Fraction B showed also a cercaricidal effect against the cercariae of *Schistosoma mansoni.* The concentrations needed to kill all *S. mansoni* cercariae (LC_{100}) within 5, 30 and 60 min were 0.7, 0.4 and 0.25 mg/l, respectively. A time-concentration relationship was observed. The cercaricidal activity of *Iris germanica* L. is in agreement with the reported data for *Millettia thonningii* (Perrett *et al.*, 1994), *Phytolacca dodecandra* (Birrie *et al.*, 1998), *Solanum nigrum* (Ahmed and Ramzy, 1997), *Jatropha curcas* (Rug and Ruppel, 2000) and *Origanum compactum* (Lahlou, 2002). This effect suggested that the flavonoids content of fraction B may also interfere with the electron transport pathway of cercariae, as reported by Lyddiard and Whitfield (2001) who surmised that the molluscicidal and cercaricidal effects of seeds of *Millettia thonningii* are due to the interference of a mixture of flavonoids in a dichloromethane extract with the electron transport systems of the isolated rat liver mitochondria.

The median lethal dose (0.5 mg/l) of fraction B showed a significant reduction of snails' heart rate after 6 to 24 h of exposure (Table III). The heart rate of examined snails after 6 h (55.2 ± 7.5) was significantly ($P < 0.001$) lower than that of the control group (65 ± 8), corresponding to a decrease of 15%. The heart rate of snails after 24 h (33.6 ± 6.3) was also significantly ($P < 0.001$) lower than that of the control group (65 ± 8), corresponding to a decrease of 48%. The heart rate of snails after 2 h (63.7 ± 7.4) did not significantly change. Copper sulphate (a known molluscicide) caused a reduction of *Biomphalaria glabrata* heart rate (Cheng and Sullivan, 1973) and the aqueous extract of a plant molluscicide, *Agave fourcryodes*, also strongly reduced the heart rate of *Biomphalaria havanensis* snails (Diaz Garces and Ferrer Lopez, 1996). It was suggested that the alterations of snails' heart rate has a relationship with the molluscicidal effect of the molluscicidal agent (Romero and Hoffmann, 1996). From these promising results, it could be concluded that the use of the widely distributed ornamental *Iris germanica* L. plant as a lead of molluscicidal agent could represent an economically viable way of vector control in Schistosomiasis endemic areas.

Table III. The effect of the median lethal dose of fraction B from the chloroform extract of *Iris germanica* L. rhizomes on the heart rate reduction of *B. alexandrina* snails.

Time after treatment [h]	Number of snails	Mean ± SD[a]	P[b]
Control	25	65 ± 8	
2	25	63.7 ± 7.4	N. S.
6	23	55.2 ± 7.5	***
24	14	33.6 ± 6.3	***

[a] SD, standard deviation.
[b] Student's *t*-test of difference between the heart rate of the snail group before and after treatment with the plant extract.
*** Significant at 0.001; N.S., not significant.

Structure determination of isolated compounds

A chromatographic study of fraction B resulted in the isolation of four compounds, of which **1** and **4** are novel (Fig. 1).

5,2′-Dihydroxy-3-methoxy-6,7-methylenedioxyflavone (**1**)

The molecular formula of **1** (Fig. 1) was determined as $C_{17}H_{12}O_7$ (M_r = 328) by high-resolution mass spectrometry (LSIMS) in the positive-ion mode, giving *m/z* 329.0657 for MH^+ (calcd. 329.0661, 1.3 ppm). UV (MeOH): λ_{max} = 215, 248, 313 nm. The ^1H NMR spectrum of **1** displayed two

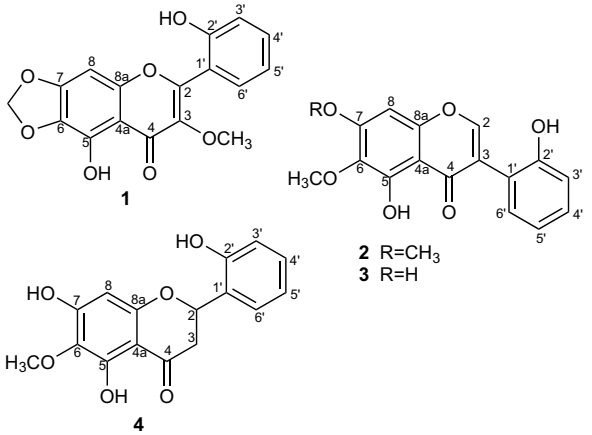

Fig. 1. Structures and the numbering of isolated compounds: 5,2′-dihydroxy-3-methoxy-6,7-methylenedioxyflavone (**1**), irilin A (**2**), irilin B (**3**) and 5,7,2′-trihydroxy-6-methoxyflavanone (**4**).

singlet signals at 6.10 ppm and 6.62 ppm with the intensity ratio 2:1. In the HSQC spectrum they showed connectivity to carbon atoms at 104.34 ppm and 90.53 ppm, respectively (Table IV). The first one is very indicative to a 6,7-methylendioxy unit and the latter fits well to the proton at position 8 (Agrawal, 1989). The proton and carbon spectra showed clearly the presence of one methoxy group and an *ortho*-hydroxy-substituted phenyl group. The H-6′ was assigned from its HMBC correlation with C-2 and from its NOESY correlation with the methoxy group, which proved also that methoxy and aryl substituents lie at the adjacent carbon atoms (Fig. 2). The rest of the aryl protons and carbon atoms were assigned based on DQF-COSY and HSQC spectra. The 2D-NMR spectral data allowed almost unambiguous assignment of all signals, but the question remained whether the structure is flavonoid or isoflavonoid. From literature data (Agrawal, 1989; Bergman *et al.*, 2001; Choudhary *et al.*, 2001), it can be found that for 3-methoxyflavonoids the chemical shifts for C-2 and C-3 are approx. 137–141 ppm and 151–158 ppm, respectively. These are in agreement with our observations. 2-Methoxy-isoflavonoid has not been observed, but for 2-unsubstituted isoflavonoids C-3 has the chemical shift of 121–123 ppm (Agrawal, 1989). If a 2-methoxy group would be added, it should shift C-3 even more upfield, which is in clear contradiction to the observation (HMBC) that the chemical shift for the carbon atom to

Table IV. NMR results for compounds **1** and **4** at 298 K in CD_3OD. Chemical shifts are expressed in ppm using TMS as a reference (0.00 ppm).

Position	δ (^{13}C) (**1**)	δ (^{1}H) (*J*, Hz) (**1**)	HMBC (H→C) correlations (**1**)	δ (^{13}C) (**4**)	δ (^{1}H) (*J*, Hz) (**4**)	HMBC (H→C) correlations (**4**)
2	156.97	–	–	76.12	5.68 dd (3.0; 13.0)	C-3; C-4; C-1′; C-2′
3	140.99	–	–	43.09	2.83 dd (3.0; 17.0)	C-4; C-4a′
					2.96 dd (13.0; 17.0)	C-2; C-4; C-1′
4	180.66	–	–	198.81	–	–
4a	109.39	–	–	103.50	–	–
5	142.63	–	–	156.61	–	–
6	131.17	–	–	130.43	–	–
7	156.09	–	–	160.53	–	–
8	90.53	6.62 s	C-4; C-4a; C-6; C-7; C-8a	96.24	6.02 s	C-4; C-4a; C-6; C-7; C-8a
8a	154.71	–	–	160.78	–	–
1′	119.09	–	–	126.78	–	–
2′	159.16	–	–	155.26	–	–
3′	117.40	6.96 dd (1.0; 8.1)	C-1′; C-2′; C-5′	116.19	6.82 dd (1.0; 8.1)	C-1′; C-5′
4′	133.31	7.37 ddd (1.7; 7.5; 8.1)	C-2′; C-6′	130.30	7.16 ddd (1.6; 7.6; 8.1)	C-2′; C-6′
5′	120.40	6.96 dt (1.0; 7.5)	C-1′; C-3′	120.69	6.88 dt (1.0; 7.6)	C-1′; C-3′
6′	131.65	7.39 dd (1.7; 7.5)	C-2; C-2′; C-4′	127.59	7.46 dd (1.6; 7.6)	C-2; C-2′; C-4′
6-OCH_3	–	–	–	61.04	3.79 s	C-6
3-OCH_3	61.17	3.74 s	C-3	–	–	–
6,7-OCH_2O-	104.34	6.10 s	C-6; C-7	–	–	–

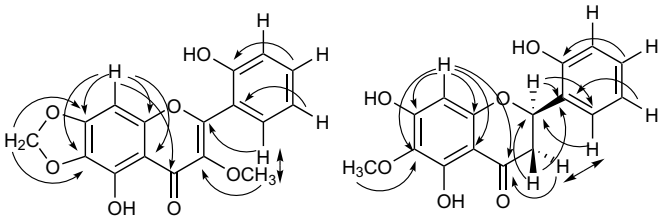

Fig. 2. Selected HMBC (→) and NOESY (↔) correlations for compounds **1** and **4**.

which an aryl group is attached is 157.0 ppm. In conclusion, the structure can be unequivocally determined as a flavonoid based on the chemical shifts of carbon atoms 2 and 3. The spectral data is very similar by its flavonoid skeleton to the one for 4′-glucosyl-5,3′-dihydroxy-3-methoxy-6,7-methylenedioxyflavone isolated from spinach extracts (Bergman *et al.*, 2001).

5,7,2′-Trihydroxy-6-methoxyflavanone (**4**)

The molecular formula of **4** (Fig. 1) was determined as $C_{16}H_{14}O_6$ (M_r = 302) by high-resolution mass spectrometry (LSIMS) in the positive-ion mode, giving *m/z* 303.0860 for MH^+ (calcd. 303.0869, 2.9 ppm). UV (MeOH): λ_{max} = 221, 287 nm. The flavanone skeleton was easily identified from the carbon chemical shifts (Table IV). Especially signals at 76.12 ppm (C-2), 43.09 ppm (C-3) and 198.81 ppm (C-4) are indicative for flavanone (Agrawal, 1989). The presence of OH at position 5 can be deduced based on the downfield shifted (because of hydrogen bonding) chemical shift of C-4. For 5-unsubstituted flavanones the C-4 shift is roughly 7–10 ppm smaller (Agrawal, 1989). Proton signals confirmed further the flavanone structure. In particular doublets of doublets at 5.68 ppm (3.0, 13.0 Hz, H-2), 2.83 ppm (3.0, 17.0 Hz, H-3eq) and 2.96 ppm (13.0, 17.0 Hz, H-3ax) are typical for flavanone. The methylene proton at 2.96 ppm can be assigned axial because of its large coupling constant with H-2. The presence of one methoxy group is obvious from proton and carbon spectra. Its position can be solved by the HMBC spectrum. A correlation can be found to the carbon atom having the chemical shift 130.43 ppm (Table IV). This value refers to the carbon atom which has hydroxy substituted adjacent carbon atoms. Therefore a methoxy group must be in position 6 and there are hydroxy groups at positions 5 and 7. The assignment is further confirmed by HMBC correlations from a proton singlet signal at 6.02 ppm (H-8). The phenyl group in position 2 must be *ortho*-hydroxy-substituted. This is clear from the shapes of the four proton signals (roughly two doublets and two triplets when only large coupling constants are concerned). H-6′ can be assigned by its HMBC and NOESY correlations (Fig. 2) and the sequence of other protons is easily revealed by DQF-COSY. Thus, the proposed structure has been constructed unequivocally by spectral data. The chemical shifts are in agreement with the ones for similar type flavanone structures found in the literature (Bhattacharyya *et al.*, 1995; Jenkins *et al.*, 1999).

Irilins A and B (**2** and **3**)

Irilins A and B have been reported previously from *Iris pseudacorus* (Hanawa *et al.*, 1991) and *Iris bungei* (Choudhary *et al.*, 2001). They were identified by their mass spectra and by comparing the NMR results with the ones from the references above.

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