Iridoid Glucosides from the Aerial Parts of *Globularia alypum* L. (Globulariaceae)

Nour-Eddine Es-Safi,*[a,b] Samira Khlifi,[c] Albert Kollmann,[a] Lucien Kerhoas,[a] Ahmed El Abbouyi,[c] and Paul-Henri Ducrot[a]

[a] *Unité de Phytopharmacie et Médiateurs Chimiques, INRA; Route de Saint-Cyr, 78026 Versailles Cedex, France:* [b] *Laboratoire de Chimie Organique et d'Etudes Physico-Chimiques, Ecole Normale Supérieure; B.P 5118 Takaddoum Rabat, Morocco: and* [c] *Laboratoire de Biochimie Appliquée et Biotechnologie, Faculté des Sciences; B.P 20 El Jadida, Morocco.* Received August 8, 2005; accepted October 18, 2005

From the hydromethanolic extract of the aerial parts of *Globularia alypum* grown in Morocco, a new chlorinated iridoid glucoside, globularioside has been isolated beside 5 known iridoid glycosides, globularin, globularicisin, globularidin, globularinin and globularimin. This is the first report of a chlorinated iridoid in *G. alypum* and in the Globulareaceae. Unlike all other known 7-chlorinated iridoid glucosides where the chlorine atom exhibits an α configuration, globularioside incorporate the chlorine atom as a 7β substituent. The structures of the isolated compounds were established on the basis of ESI-MS, MS-MS, 1D and 2D NMR spectral analysis.

Key words *Globularia alypum*; Globulariaceae; iridoid; iridoid glucoside; chlorinated iridoid; globularioside

Globularia alypum L. is a wild plant belonging to Globulariaceae family. It is a perennial shrub which is found throughout the mediterranean area. The plant is known for its uses in the indigenous system of medicine for a variety of purposes.[1)] In the Moroccan Traditional Pharmacopoeia, *G. alypum* locally named "Ain Larneb" is one of the most traditional plant remedies.[2)] Its leaves are traditionally used as hypoglycaemic agent, laxative, cholagogue, stomachic, purgative and sudorific.[3)] It is also used in the treatment of cardiovascular and renal diseases as demonstrated by a recent ethnobotanical surveys, which showed that *G. alypum* is one of the most used medicinal plant in Morocco.[2,4)] The infusion of *G. alypum*, exhibiting no toxicological effects, was thus shown to produce a significant hypoglycaemic in rats both by oral and intraperitoneal administration.[5)] A significant antileukemic activity of an aqueous extract of *G. alypum* was also reported.[6)] Recently, methanol and dichloromethane extracts of *G. alypum* were also shown to reduce histamine and serotonin contraction *in vitro*.[7)]

As part of our continuing search of bioactive natural products, we investigated the iridoids of *G. alypum* grown in Morocco. The wide use of this plant for the treatment of many diseases in addition to the fact that no phytochemical study is reported on the Moroccan *G. alypum* strain prompted us to explore its chemical composition related to its antioxidative activity. So far, the only chemical investigations of *G. alypum* are those of Bernard *et al.*,[8,9)] Chaudhuri and Sticher[10—12)] and Ben Hassine *et al.*[13)] where the presence of some glycosidic iridoids, flavonoids and a lignan diglucoside was reported. Our preliminary investigations on the biological activity of this plant showed that the hydro-methanolic extract of its aerial parts exhibited significant antioxidant effect, based on the scavenging activity of the stable 2,2-dipenyl-1-picrylhydrazyl (DPPH) free radical. We report here the isolation and the structure elucidation of a new chlorinated iridoid named globularioside along with 5 known iridoids by ESI/MS, MS-MS, 1D and 2D NMR analysis.

Experimental

General Experimental Procedures UV–visible spectra were recorded using a Kontron Uvikon 930 spectrophotometer fitted with a quartz cell. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. FT-IR spectra were recorded with a Nicolet Avatar 320 FT-IR spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded in CD_3OD with a Varian Mercury plus 300 spectrometer at 300 and 75 MHz, respectively for ^{1}H and ^{13}C (broad band proton decoupling mode for carbon). Analytical TLC was performed on Merck silica gel 60 F254 plates. Column chromatography was performed on SPE column using mixtures of MeOH/Water. Analytical HPLC analysis was performed on a Varian apparatus including a 9012 solvent delivery system, a 9100 autosampler and a 9065 polychrom diode array detector. Analysis were performed on a Kromasil reversed phase C18 5 μm column (250×4.6 mm) eluting with a mixture of solvents A: acetonitrile and B: water with 0.5% acetic acid eluting from 15 to 100% A in 18 min followed by a washing and a reequilibrating of the column. Isolation of the studied compounds was performed on a semipreparative HPLC apparatus including a Millipore Waters 600 Multisolvent Delivery system, a Waters U6K manual injector and a TSP-UV200 Dual-Wavelength UV/visible programmable detector. The separations were performed on a Kromasil C18 10 μm column (250×20 mm) eluting with a mixture of solvents A: acetonitrile and B: water with 0.5% acetic acid eluting from 5 to 30% A in 120 min followed by a washing and a reconditioning of the column.

LC/MS analysis were performed with a chromatographic system (Alliance) including a Waters 2695 separations module equipped with an autosampler and a Waters 2487 dual lambda absorbance detector (Waters, Milford, MA, U.S.A.). The separation was achieved at a flow rate of 200 μl/min on a Uptisphere 5ODB RP18 column (5 μm packing, 150×2 mm I.D.) protected with a guard column of the same material (Interchim, Montlucon, France). Analysis were performed at a temperature of 24 °C, samples were kept at 10 °C and a volume of 5.0—10.0 μl was injected for each analysis. The elution was done with solvents A (H_2O 0.5% AcOH) and B (CH_3CN 0.5% AcOH) and the conditions were adapted as follows: linear gradients from 10 to 80% B in 30 min and from 80 to 100% B in 20 min, followed by washing and reconditioning of the column. The HPLC system was coupled on line to an ESI-MS instrument into which the effluent from the UV detector was introduced without any split. ES mass spectrometry and MS-MS experiments were performed on a Quattro LC™ micromass spectrometer with a Z-spray™ ES source (Waters, Milford, MA 01757, U.S.A.). The ES source potentials were: capillary 3.25 kV (positive mode) or 3.0 kV (negative mode), extractor 2.0 V and RF Lens 0.1 V. The sampling cone voltage was varied from 20 to 50 V for ES mass spectra and the specific value of the cone voltage for each collision-induced dissociation (CID) experiment were set in order to optimize the parent ion. The quadruple mass filters were set with LM and HM resolution of 15.0 (arbitrary units), which is equivalent to 1.0 Da mass window for transmission of both precursor and product ions. The source block and desolvation temperatures were set at 120 and 400 °C respectively. Nitrogen was used as drying, nebulising and cone gas. Argon was used as collision gas at 3.5×10^{-3} mbar. Data acquisition and processing were carried out using software MassLynx version 4.0. Compounds were in-

* To whom correspondence should be addressed. e-mail: nouressafi@yahoo.fr

troduced using a Waters 2695 separation module (Alliance). For direct injection, the solution was introduced into the electrospray source with a medical syringe infusion pump (Harvard Apparatus, Holliston. MA, U.S.A.) in combination with a 100 μl syringe.

Plant Material Fresh aerial parts of *Globularia alypum* L. was collected from Taza region, Morocco in April 2003. Taxonomic identification was performed by Dr. R. Tellal, Department of Biology, University of El Jadida Morocco. A voucher specimen (KS_2) has been deposited in the Herbarium of the Department of Biology, Faculty of Sciences, Hassan II University, El Jadida, Morocco.

Preparation of Plant Extract Fresh aerial parts were air-dried in shade at room temperature and the dried aerial parts were powdered. 100 g of the obtained powder were macerated during 48 h at room temperature with 500 ml of mixture of distilled water–methanol (3/2). The crude preparation was filtered and concentrated under reduced pressure to provide a crude extract (10.75 g) which was stored at −20 °C until use.

Extraction and Isolation The crude extract was further eluted in water and the obtained aqueous phase was extracted with hexane and the further aqueous phase was subjected to a SPE column. Elution was performed successively by H_2O, MeOH 10%, MeOH 40%, MeOH 50%, MeOH 70% and MeOH 100%. The obtained fractions were concentrated under reduced pressure, lyophilised tested for their scavenging activity and analysed through analytical HPLC. The MeOH 50% fraction was shown to be rich in natural antioxidant compounds was explored first by semipreparative HPLC. After several successive injections, samples corresponding to the same chromatographic peaks were controlled by analytical HPLC, concentrated under reduced pressure and lyophilised. This operation gave pure iridoids **1**—**6**.

Reduction of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical[14] TLC autographic Assay: Methanolic solutions (0.1%) of the isolates were chromatographed on a silica gel plate using $CHCl_3$/MeOH/H_2O (61/32/7) solvent system. After developing and drying, TLC plates were sprayed with a 0.2% DPPH solution in MeOH. Compounds showing a yellow on purple spots were regarded as antioxidant.

Spectrophotometric Assay: Fifty microliters of a solution containing the compound to be tested were added to 5 ml of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min, and percent of activity was calculated by comparison with a control sample.

Globularioside (**6**): Amorphous solid, $[\alpha]_D^{20}$ −25° (c=0.01, MeOH). ^{1}H-NMR (CD_3OD, 300 MHz) and ^{13}C-NMR (CD_3OD, 75 MHz) spectroscopic data: see Table 1. HR-MS m/z: 529.1433 $[M+H]^+$ (Calcd for $C_{24}H_{30}ClO_{11}$: 529.1477). ESI-MS and MS-MS results: see Figs. 2 and 3.

Results and Discussion

Air dried aerial parts of *G. alypum* were extracted with a water/methanol (3/2) mixture. After filtration and evaporation of methanol, the aqueous phase was extracted with hexane to remove non-polar substances. The further obtained aqueous solution was subjected to a SPE column chromatography using a step gradient of MeOH/H_2O mixture (0/100, 10/90, 40/60, 50/50, 70/30, 100/0) and each fraction was tested for its antioxidant activity. The obtained results revealed that the 50% aqueous methanolic fraction exhibited an interesting antioxidant activity as attested by its scavenging activity of the stable DPPH free radical, and was thus further investigated for its phytochemical composition. Analytical HPLC-DAD analysis of this fraction revealed the presence of various iridoid and phenolic derivatives. This fraction was lyophilized and subjected to a reversed phase semi-preparative HPLC giving a number of pure iridoids, phenyl ethanoid glucosides and flavonoids. All isolates were tested for their radical scavenging activity using DDPH. Phenylethanoid and flavonoid glycosides were found to possess potent antioxidant activity compared to that of iridoids. Among the isolated compounds, five known iridoids globularin **1**, globularicisin **2**, globularidin **3**, globularinin **4** and globularimin **5** in addition to a new chlorinated iridoid glucoside **6**, which we named globularioside (Fig. 1) were isolated and their structures established on the basis of spectral analysis and comparison with literature data.[10—12,15]

In addition to *G. alypum*, globularin **1** has also been isolated from *G. davisiana*,[16] *G. sintenisii*[17] and *G. orientalis*.[18] Globularicisin **2** and globularidin **3** were respectively reported in *G. davisiana*[16] and *G. trichosantha*.[19] It is therefore possible that such iridoids are common in the family of Globulareaceae.

Beside these known substances, a new iridoid **6** was isolated as an amorphous powder. The molecular formula was determined as $C_{24}H_{29}ClO_{11}$ by both positive and negative ESI-MS (m/z: 527 $[M-H]^-$, 563 $[M+Cl]^-$, 529 $[M+H]^+$, 551 $[M+Na]^+$). The UV spectrum exhibited maxima at 217 and 279 nm. Its IR spectrum showed absorptions at 3403, 1698, 1635, and 1450 cm^{-1} indicating OH, ester C=O functionalities and aromatic C=C bonds. Further spectroscopic

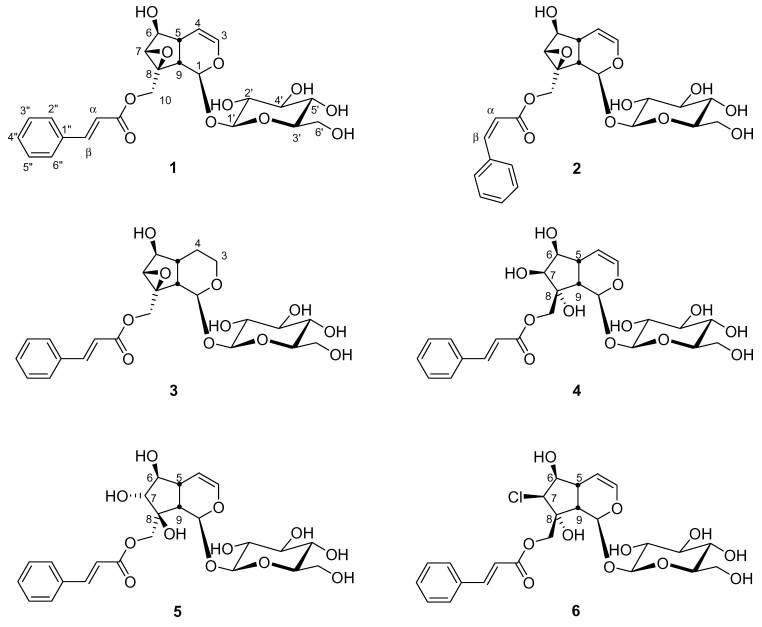

Fig. 1. Structure of the Six Iridoids Isolated from *G. alypum*

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data (in CD_3OD) of the New Iridoid Glucoside **6** Isolated from *G. alypum*

Position	DEPT	δ_H (ppm)	δ_C (ppm)	m	J (Hz)
1	CH	5.63	96.5	d	3.6
3	CH	6.22	141.7	dd	1.5, 6.0
4	CH	5.06	106.7	dd	3.4, 6.0
5	CH	2.70	39.9	m	—
6	CH	3.94	80.5	dd	3.6, 7.2
7	CH	4.08	67.2	d	7.2
8	C	—	84.5	—	—
9	CH	2.63	44.6	dd	3.6, 9.9
10a	CH_2	4.32	66.6	d	12.0
10b	CH_2	5.06	66.6	d	12.0
1′	CH	4.75	101.3	d	7.8
2′	CH	3.18	75.7	m	—
3′	CH	3.30	79.6	m	—
4′	CH	3.69	72.3	m	—
5′	CH	3.69	80.0	m	—
6′a	CH_2	3.65	63.6	dd	5.8, 11.8
6′b	CH_2	3.89	63.6	dd	2.0, 11.8
1″	C	—	136.6	—	—
2″	CH	7.62	130.9	br s	—
3″	CH	7.41	130.1	br s	—
4″	CH	7.40	132.4	br s	—
5″	CH	7.41	130.1	br s	—
6″	CH	7.62	130.9	br s	—
C(α)	CH	6.53	147.5	d	16.2
C(β)	CH	7.73	119.6	d	16.2

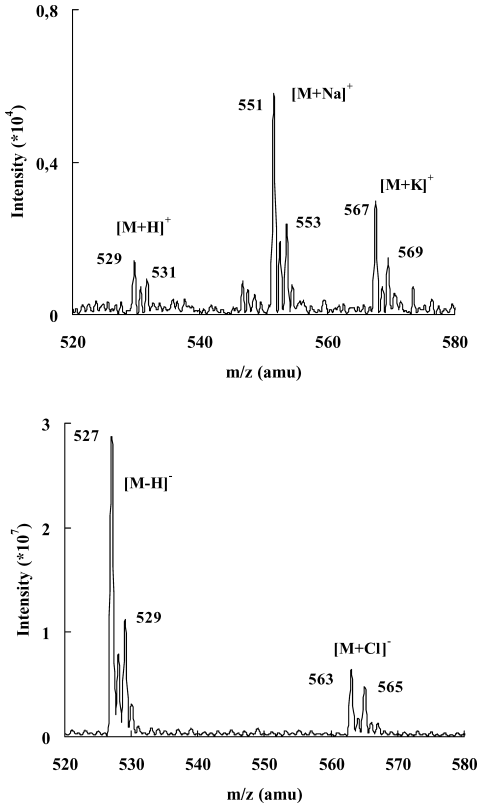

Fig. 2. Positive and Negative ESI-MS Spectra of Globularioside **6** Showing the Chlorine Atom Characteristic Isotopic Mass Pattern

data allowed us to establish its structure as the chlorinated iridoid **6** shown in Fig. 1. The MS-MS analysis showed indeed in particular a loss of a portion corresponding to a mass of *m*/*z*: 162 amu suggesting as for compounds **1**—**5** the presence of a sugar moiety in the molecule. This was confirmed in the ^{13}C-NMR spectrum, which, in addition to the characteristic glucopyranosyl unit, revealed the presence of one cinnamoyl moiety with a mono-substituted aromatic ring, in addition to the aglycone moiety containing nine carbon signals (Table 1).

The ^{1}H-NMR spectrum of compound **6** displayed signals in agreement with an acylated iridoid glycoside. Preliminary assignment of the proton chemical shifts were obtained from the homonuclear ^{1}H–^{1}H correlated spectroscopy. The anomeric proton resonance at 4.66 ppm and the signals in the region 3.20—3.86 ppm together with the corresponding C-resonances indicated the presence of a β-glucopyranosyl unit. The resonances of the two olefinic protons of the cinnamoyl moiety were readily identified as two doublets resonating downfield at 6.53 and 7.73 ppm respectively, with a large coupling constant (J=16.2 Hz) in agreement with a *trans* stereochemistry. In addition, the presence of five aromatic protons [7.62 ppm (2H), 7.41 ppm (3H)] was furthermore consistent with the presence of an (*E*)-cinnamoyl moiety. The ^{13}C-NMR spectrum of **6** exhibited 24 signals (Table 1); six of them were attributed to a β-D-glucopyranosyl unit as confirmed through hydrolysis followed by chromatography, while nine of them were ascribed to an (*E*)-cinnamoyl moiety. All the remaining C-resonances indicated that **6** had an iridoid skeleton with nine carbon atoms.

For the analysis of the iridoid skeleton, the starting point was the resonance at 6.22 ppm (dd, J=6.0, 1.5 Hz) which was assigned to H-3. This signal showed correlations with the signals located at 5.06 and 2.70 ppm, which were therefore assigned to H-4 and H-5 respectively. The proton H-5 signal exhibited correlations with protons located at 2.63 and 3.94 ppm which were then assigned to H-9 and H-6. The signal located at 4.08 was assigned to H-7 through its correlation with the signal located at 3.94 ppm previously attributed to H-6 and the last signal located at 5.63 ppm was attributed to H-1 through its correlation with the H-9 proton. The H-10 protons were easily assigned to the AB spin system located at 4.32 and 5.06 ppm with a large coupling constant (J= 12.0 Hz).

It must be noted that ^{1}H- and ^{13}C-NMR spectral data of compound **6** showed a great similarity with those of compound **4**. The main difference between compound **4** and **6** is the presence of carbon signal at 67.2 instead of 78.6 ppm corresponding to the C-7 resonance. This upfield shift ($\Delta\delta$=−11.4 ppm) suggested different substitution pattern at C-7. Similar behavior was observed for the previously reported 7-chlorinated iridoids.[20—22]

The presence of the chlorine atom was unambiguously confirmed through ESI-MS analysis conducted both in positive and negative-ion mode where the characteristic isotopic pattern mass was observed. In negative-ion mode, the mass spectrum exhibited a signal corresponding to the ion $[M-H]^-$ at *m*/*z* 527 and 529 (isotope peak) (approx. 3 : 1). The same characteristic was observed when the analysis was conducted in the positive ion mode and where two peaks in a 3 : 1 ratio were observed respectively at *m*/*z* 529 and 531 (isotope peak) corresponding to the $[M+H]^+$ ion (Fig. 2). Figure 3 shows the main fragmentations obtained through MS-MS analysis with or without the loss of the chlorine atom.

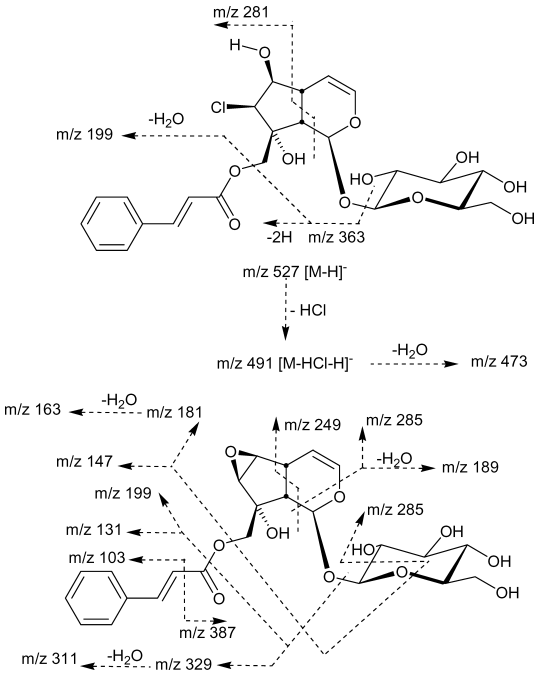

Fig. 3. Proposed Negative Ion Collision-Induced Dissociation (CID) Fragmentations of Globularioside (*m*/*z* 527) and Its Corresponding Dehydrochlorided Adduct (*m*/*z* 491)

The relative configurations of the stereogenic centers in **6** were determined from the different coupling constants observed in the ^{1}H spectrum and from a NOESY experiment. The β substitution at C-1 was established through observation of a coupling constant of 3.6 Hz between H-1 and H-9 which is in agreement with a β configuration of these two protons in a *cis* catalpol ring fusion. The *cis* ring junction was supported by the large coupling constant between H-5 and H-9 (9.9 Hz) as well as the correlation of these two protons in the NOESY spectrum. A further correlation was observed between H-6 and H-7 indicating their *cis* orientations. The coupling constants between H-5/H-6 (J=3.6 Hz) and H-6/H-7 (J=7.2 Hz) supported respectively *trans* and *cis* configurations of these protons, which are further supported by the fact that no correlations were found between H-6 and H-5 nor H-9 in the NOESY data. The setereochemistry of the C-8 center was determined to be α OH and β CH_2OR since there was good agreement between the C-9 resonance value with compound **4**. These data confirmed the stereostructure of **6** as shown in the Fig. 1. To our knowledge, compound **6** is described here for the first time and named globularioside.

Globularioside **6** belongs to a group of chlorine containing iridoids, the number of which has been steadily increasing. Each of the known chlorinated iridoids are chlohydrins derived from an iridoid epoxide, consistently present in the same plant. It seems therefore safe to assume that the known members of this class of iridoids are produced in the plant by addition of chloride ion to the corresponding iridoid epoxide.

This study showed that iridoids represent a large and still expanding group of monoterpenoids. They are found as natural constituents in a large number of plant families, usually, as glucosides. Iridoid glucosides have biogenetic and chemotaxonomic importance since they provide a structural link between terpenes and alkaloids. In some instances, the presence of iridoids has been used to support a defined botanical classification. In the family of globulareaceae, iridoids are widespread metabolites with chemotaxonomic significance. *Globularia* species have been known to be rich in iridoid derivatives and various free and acylated iridoids were reported in *G. alypum*,[10—12] *G. davisiana*,[16] *G. sintensii*,[17] *G. orientalis*,[18] *G. trichosantha*,[19] *G. dumulosa*[23] and *G. cordifolia*.[24,25] However, chlorinated iridoids have never been reported before from globularia genus. The existence of such compounds in a *Globularia* specie might be of great chemotaxonomical importance at both the family and the genus levels.

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