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Sesquiterpenoids from *Leontodon tenuiflorus* (Asteraceae, Cichorieae): First record of a hypocretenoid from *Leontodon* section Asterothrix

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Keywords: *Leontodon*; Cichorieae; Hypochaeridinae; Asteraceae; *Sesquiterpene lactones*; Phenolics; Chemophenetics.

ABSTRACT

Leontodon tenuiflorus (Gaudin) Rchb. yielded ixerisoside D, sonchuside A , and the previously undescribed 4,6-O-dihypocretenoyl-D-glucopyranoside. This is the first report of a hypocretenoid from Leontodon section Asterothrix.

1. Subject and source

Leontodon tenuiflorus (Gaudin) Rchb. [synonym: *Leontodon incanus* (L.) Schrank subsp. *tenuiflorus* (Gaudin) Schinz & R.Keller] is an herb of 10–40 cm height. The taxon grows in rock fissures, scree, and sun exposed steep slopes at the Southern margins of the Alps, from the Swiss canton of Ticino to Monte Baldo and in the alpine foothills of Friuli (Zidorn 2012, 2018).

L. tenuiflorus was grown in the Botanical Garden of the Department of Pharmaceutical Biology (N 54◦19′ 59.9″ , E 10◦06′57.5’’; 35 m a.m.s.l.) from seeds collected in the wild near Borno between the Rifugio Laeng and the Pizzo Camino (N 45◦58′49.3″ , E 10◦11′ 14.2″ , alt.: 1870 m a.m.s. l., Brescia, Lombardia, Italy; CZ-20170814D-1). Plants in the garden of the Pharmaceutical Institute of Kiel University were harvested in September 2018 and a voucher specimen was deposited in the Herbarium of Kiel University (CZ-20180917A-1, KIEL0005013). The plants were determined using the keys and characters provided by Zidorn (2012).

2. Previous work

There are no dedicated previous studies on the chemistry of *L. tenuiflorus*. In a comparative study on the phenolics of flowering heads of various taxa of the genus *Leontodon* s.l., Zidorn et al. (1998) detected aesculin, chlorogenic acid, 3,5-*O*-dicaffeoylquinic acid, caffeoyltartaric acid, cichoric acid, luteolin, luteolin 7-*O*-glucoside, luteolin 7-*O*-gentiobioside, luteolin 7-*O*-glucuronide, and luteolin 4′ -*O*-glucoside in flowering heads of *L. tenuiflorus* from the Province of Trento/Italy. In flowering heads of the species closest related to *L. tenuiflorus*, *L. incanus* (L.) Schrank, chlorogenic acid, 3,5-*O*-dicaffeoylquinic acid, 4, 5-*O*-dicaffeoylquinic acid, caffeoyltartaric acid, cichoric acid, luteolin, luteolin 7-*O*-glucoside, luteolin 7-*O*-gentiobioside, luteolin 7-*O*-glucuronide, and luteolin 4′ -*O*-glucoside were detected (Zidorn et al., 1998; Zidorn, 2008a). In L. *crispus* Vill. s.str., also a

member of *Leontodon* section Asterothrix, aesculin, chlorogenic acid, 3,5-*O*-dicaffeoylquinic acid, luteolin, luteolin 7-*O*-β-D-glucuronide, phaseolic acid, and equisetumpyrone were identified (Zidorn et al., 2006). However, sesquiterpene lactones or their immediate precursors have not been detected in any member of *Leontodon* section Asterothrix yet.

3. Present study

Air dried and ground plant material (284 g) was exhaustively extracted with acetone and then with a mixture of acetone/MeOH/H_2O (3/1/1) at room temperature, obtaining 19.3 g and 18.2 g of extract, respectively. These two extracts were re-dissolved in a mixture of acetone and water and partitioned with ethyl acetate yielding 7.55 g and 1.50 g of dry ethyl acetate layers after evaporation *in vacuo*, respectively. TLC analyses showed that these two ethyl acetate layers showed similar composition. Therefore, they were combined. RP-HPLC analysis of the combined phases (instrument: Hitachi ChromasterUltra Rs; column: Luna Omega 1.6 µm C18; 100 × 2.1 mm: injection volume 5.0 µl; flow rate: 0.300 ml/min; oven temperature: 30 °C; detection wave lengths: 210 nm, 254 nm, 280 nm and 360 nm; solvent A: H_2O, solvent B: MeCN; linear gradient (% B): from 5% to 95% in 45 min, 60 min: 95%) at 210 nm yielded the chromatogram in Fig. 1. The combined ethyl acetate layers (9.05 g) were fractionated by medium pressure silica gel (Büchi PrepChrom C-700) using a gradient from pure CH_2Cl_2 to pure MeOH as eluent, obtaining nine fractions (F1–F9). The residue of F3 (1.44 g) was fractionated by medium pressure silica gel (Büchi PrepChrom C-700) using a gradient from 100% *n*-hexane to 100% EtOAc and then to 100% MeOH as eluent, yielding twelve fractions (F3.1-F3.12). The residue of F3.5 (132.5 mg) was further fractionated by Sephadex LH-20 chromatography using acetone as eluent, followed by preparative medium pressure liquid chromatography (MPLC), employing a Phenomenex Aqua 5 µm C18 125 Å 50 × 10 mm, using H_2O (A) and MeCN (B) as eluents with a flow rate of 2.0 ml/min, column temperature of 30 °C and the following gradient: 50% B to 95% B in 10 min, yielding pure compounds **4** (2.6 mg) and **5** (8.8 mg). The residue of F3.7 (65.3 mg) was purified by MPLC using the same method, again yielding compounds **4** (5.2 mg) and **5** (1.3 mg). The residue of F5 (58.1 mg) was purified by MPLC using H_2O (A) and MeCN (B) with a flow rate of 2.0 ml/min, column temperature of 35 °C and the following gradient: 30% B to 45% B in 20 min, and to 95% B in 10 min, yielding pure compounds **1** (1.0 mg), **2** (1.2 mg), and **3a**/**3b** (1.4 mg). The residue of F6 (142 mg) was purified with a Sephadex LH-20 chromatography using MeOH as eluent and then a MPLC using the same method as used for F5, again yielding compounds **1** (2.6 mg), **2** (0.9 mg), and **3a**/**3b** (2.5 mg). Peaks assignable to **3a** and **3b**

where well separated both in analytical as well as in semi-preparative HPLC. However, after re-dissolving both separately collected compounds in methanol, HPLC analyses showed again two peaks in the same ratio of about 6:4 (**3a**/**3b**). In the following, we therefore analyzed the interconverting compound pair together.

Isolated compounds **1** and **2** were identified by their on-line ESI-MS spectra and NMR spectra measured in CD_3OD and in comparison with published data (Zidorn, 2008b; Shulha and Zidorn, 2019) as ixerisoside D **1** (Warashina et al., 1990) and sonchuside A **2** (Miyase and Fukushima, 1987; Zidorn et al., 2005) (Fig. 2). Compounds **4** and **5** were identified by their on-line mass spectra and NMR spectra measured in $CDCl_3$ and in comparison with published data as linolenic acid **4** (Lainer et al., 2020) and linoleic acid **5** (Lainer et al., 2020). HRMS of compound **3** revealed a molecular formula of $C_{36}H_{44}O_{12}$ [*m/z* = 691.2773 $[M + Na]^+$ (calcd. 691.2731), 669.2953 $[M + H]^+$ (calcd. 669.2911), 633.2750 $[M - 2H_2O + H]^+$ (calcd. 633.2700)]. Close inspection of NMR data showed that the analyte consisted of an approximately 1:1 mixture of a diacylated α- and β-glucopyranoside (Roslund et al., 2008), respectively. 1D and 2D-NMR experiments proved that the two acyl moieties were identical and that they consisted of sesquiterpenoyl moieties. These sesquiterpenoyl moieties were identified as hypocretenoyl moieties by ^1H NMR, ^{13}C NMR, COSY, HSQC, and HMBC experiments and by comparing the obtained spectra with the literature data of the next similar compounds already described, i.e. methyl hypocretenoate, hypocretenolide (Bohlmann and Singh, 1982), and the hypocretenoate moiety of 14-hydroxyhypocretenolide-β-D-glucopyranoside-4′ ,14″ -hydroxyhypocretenoate (Zidorn et al., 1998). ^1H NMR shift values and HMBC crosspeaks furthermore showed that the hypocretenoyl moieties were attached to the glucose core of the molecule in positions 4 and 6 (Fig. 3). The absolute configuration of the sugar moiety of compound **3** was determined after hydrolysis and octylation using the solution returned from the successive NMR measurements, employing the method described in detail in Uddin et al. (2020). The sugar moiety was thus identified as D-glucose. NMR data of the compound, which was conclusively identified as 4,6-*O*-dihypocretenoyl-D-glucopyranoside, are displayed in Table 1. The compound is described here for the first time and might be interpreted as a precursor in the biosynthesis of other hypocretenolide derivatives. No additional physical data (m.p., IR spectra, optical rotation, etc.) for 4,6-*O*-dihypocretenoyl-D-glucopyranoside were measured, because after measuring NMR spectra on in total three different instruments (400, 500, and 600 MHz), HPLC analysis of compound **3** showed clear signs of decomposition [i.e. additional signals assignable to hypocretenolide (Bohlmann and Singh, 1982) and tentatively assigned monohypocretenoyl-D-glucopyranosides]. In order to assure

that sesquiterpenoids found in the sample of *L. tenuiflorus* from cultivated plants were also present in plants collected from the wild and in order to check whether these compounds are restricted to *L. tenuiflorus* or also occur in closely related taxa, we performed a comparative chemophenetic study analyzing one sample of *L. biscutellifolius* DC., two samples of *L. hispidus* L., six samples of *L. incanus*, and six samples of *L. tenuiflorus* for the occurrence of compounds **1**–**3**. Analytical methods were established previously (Willer et al., 2020). Samples were assigned according to Zidorn (2012). The following samples (all collected and identified in the flowering state) were analyzed (most sample numbers contain the collection date) by UHPLC-MS: *L. biscutellifolius*, SE Psarades, CZ-20130519B-4, Florina, Macedonia, Greece, N 40°49′27.1″ , E 21°03′07.6″ , 1060 m a.m.s.l.; *L. hispidus*, CZ-20161002A-1, Kloster Preetz, Pl¨on, Schleswig-Holstein, Germany, N 54°14′ 27.5″ , E 10°17′02.7″ , 27 m a.m.s.l.; *L. hispidus*, CZ-20180708C-1, NE Rino di Sonico, Lombardia, Italy, 740 m a.m.s.l.; *L. incanus*, CZ-L095, between Rucorvo and Rivalgo, Belluno, Veneto, Italy, N 46°21′34″ , E 12°20′30″ , 31.05.1997, 490 m a.m.s.l.; *L. incanus*, CZ-99-00379, Kobenzl near Mödling, Mödling, Lower Austria, Austria, N 48°05′06″ , E 16°16′41″ , 30.04.1999, 290 m a.m.s.l.; *L. incanus*, CZ-20030524A-1, above Tramin, Bozen, Trentino-Südtirol, Italy, N 46°20′37.2″ , E 11°14′05.6″ , 400 m a.m.s.l.; *L. incanus*, CZ-20030529A-1, Zirl, above the vineyard, Innsbruck-Land, Tirol, Austria, N 47°16′27.2″ , E 11°15′ 16.6″ , 680 m a.m.s.l.; *L. incanus*, CZ-20030529B-1, near Brand above Telfs, Innsbruck-Land, Tirol, Austria, N 47°19′ 07.8″ , E 11°06′06.4″ , 760 m a.m.sl.; *L. incanus*, CZ-20040523A-1, Furglau, above Gasthof Steinegger, near St. Michael, Bozen, Trentino-Südtirol, Italy, N 46°27′ 11.6″ , E 11°13′17.9″ , 1070 m a. m.s.l.; *L. tenuiflorus*, CZ-20040521A-1, N Lago di Ledro between Mezzolago and Molina di Ledro, Trento, Trentino-Südtirol, Italy, N 45°52′50.3″ , E 10°45′33.1″ , 670 m a.m.s.l.; *L. tenuiflorus*, CZ-20040521B-1, NE Lago d'Idro between Bondone and the Rifugio Alpo, Trento, Trentino-Südtirol, Italy, N 45°48′19.3″ , E 10°34′11.6″ , 1120 m a.m.s.l.; *L. tenuiflorus*, CZ-20040522A-1, between Tavernole and Dosso di Marmentino, Brescia, Lombardia, Italy, N 45°45′ 00.8″ , E 10°15′12.0″ , 640 m a.m.s.l.; *L. tenuiflorus*, CZ-20140529A-1, Molina di Ledro, below the cliffs W Val d'Oro, Trento, Trentino-Südtirol, Italy, N 45°52′41.1″ , E 10°46′48.6″ , 970 m a.m.s.l.; *L. tenuiflorus*, CZ-20140608A-1, Barzizza (Gandino) along the road to Monte Farno, Bergamo, Lombardia, Italy, N 45°49′31.7″ , E 09°54′27.7″ , 930 m a.m.s. l.; *L. tenuiflorus*, CZ-20140713B-1, Valcamonica, Cimbergo, above Rifugio de Marie al Volano, Brescia, Lombardia, Italy, N 46°01′ 19.2″ , E 10°24′19.1″ , 1570 m a.m.s.l.

HPLC-DAD-ESI-MS results are summarized in Table 2. Based on findings from pure compounds isolated in the course of this study, ixerisoside D **1** was assigned in crude extracts by peaks at R_t = 13.7 min showing signals at *m/z* = 409 $[M + H]^+$ in the positive and at *m/z* = 453 $[M + HCOOH - H]^-$ in the negative mode, respectively. Sonchuside A **2** was assigned by peaks at R_t = 12.9 min showing signals at *m/z* = 431 $[M + NH_4]^+$ and 435 $[M + Na]^+$ in the positive and at *m/z* = 411 $[M - H]^-$ and *m/z* = 457 $[M + HCOOH - H]^-$ in the negative mode, respectively. Accordingly, compounds **3a** and **3b** were assigned by peaks with retention times of R_t = 15.3 min and 15.7 min and identical mass signals at *m/z* = 633 $[M - 2H_2O + H]^+$ and *m/z* = 713 $[M + HCOOH - H]^-$ in the positive and in the negative mode, respectively.

UHPLC-MS results have to be interpreted with some caution, because all analytical samples were retrieved from vouchers from the private herbarium of CZ. Therefore, all samples stem only from one plant and the samples had not been dried in a way to optimize for the preservation of natural products (e.g. freeze-drying or at least fast drying of the tap roots of members of section Asterothrix by splitting them in two halves before drying). This fact (rather than some intra-specific variations) might partially explain some of the findings outlined below. Another factor to be taken into consideration is that some of the investigated samples were quite old (the oldest was collected in the year 1997), therefore some natural products previously present in the samples might have decomposed in the meantime.

Ixerisoside D **1** was detected in samples of all investigated species, but not in all samples; in each case it was detected, it was a minor compound based on the HPLC-UV traces recorded at λ = 230 nm. Sonchuside A **2** was only detected (in low amounts) in one sample of *L. incanus* and the two samples of *L. hispidus*. The newly described hypocretenolide precursor compound **3**, the actual reason for this comparative phytochemical investigation, was not detected in the samples of *L. hispidus*; in contrast, the compound was detected in the sample of *L. biscutellifolius*, two out of six samples of *L. incanus*, and three out of six samples of *L. tenuiflorus*.

4. Chemophenetic significance

The phylogenetic relationships of *Leontodon* section Asterothrix, of which *L. tenuiflorus* is a member (Widder, 1975; Zidorn, 2012), are shown in Fig. 4. The displayed tree topology is a based on a maximum likelihood phylogram reported by Enke et al. (2012). Based on the preliminary survey described above and based on the fact that in previous extensive investigations of *L. hispidus*,

compound **3** was never detected, the newly discovered sesquiterpenoid **3** might be a chemophenetic marker of the section Asterothrix (Fig. 4). The fact that compound **3** was detected in some but not all samples of *L. incanus* and *L. tenuiflorus* might, as indicated above, either be explained by an intra-specific variation within *L. incanus* or by the chemical instability of the compound. In contrast, compound **3** was not found in the two investigated samples of *L. hispidus*. For the moment, compound **3** has therefore only been detected in members of *Leontodon* section Asterothrix. However, the most important finding of the current study is that hypocretene derivatives, i.e. guaian-12,5-olides and their precursors such as hypocretenoic acid and compound **3**, now have also been found in *Leontodon* section Asterothrix. So far, this class of compounds had only been found in *Hypochaeris cretensis* (L.) Bory & Chaub. (they were actually named after this species) and members of *Leontodon* section Leontodon (Enke et al., 2012); in contrast, an additional previous record from *Crepis aurea* (L.) Cass. was proven to be erroneous (Michalska et al., 2013). While preparing this manuscript, we tried to verify the identity of the plant material the first report of hypocretenolides from *H. cretensis* was based on (Bohlmann and Singh, 1982). The plants for that paper were cultivated in Berlin from seeds obtained from the Botanical Garden of Dijon/France (the source of these plants was not revealed). Unfortunately, the Bohlmann herbarium seems to be lost (written messages from Dr. Norbert Kilian, BGBM, Berlin and Dr. Lars Merkel and Professor Roderich Süßmuth, Technical University of Berlin). Given the unique structure of hypocretenolides, the so far extremely restricted occurrence of this compound class, the proven unreliability of the seed exchange service between botanic gardens, and the known difficulties involved in reliably determining yellow flowering members of the Cichorieae tribe (not by chance referred to by some botanists as DYCs for “damned yellow composites”), we are tempted to assume that the first hypocretenolide had actually not been isolated from *H. cretensis,* but from a superficially similar member of the genus *Leontodon*, e.g. *L. cripus* Vill. s. l. To further test this hypothesis we will perform additional studies in the future.

CRediT authorship contribution statement

Roberta Di Lecce: Methodology, Investigation. **Mayra Galarza P´erez:** Methodology, Investigation, Writing – review & editing. **Alfonso Mangoni:** Writing – review & editing. **Serhat Sezai Çiçek:** Supervision, Writing – review & editing. **Frank D. S¨onnichsen:** Methodology, Writing – review & editing. **Antonio Evidente:** Supervision, Writing – review & editing. **Christian**

Zidorn: Conceptualization, plant collections, Writing – original draft, Writing – review & editing, Supervision, Project administration, Formal analysis, data analyses, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors wish to thank Dr. Ulrich Girreser and his team of the NMR Unit of the Pharmaceutical Institute of Kiel University for expert NMR support. Special thanks go to the gardeners of the Botanical Garden of the Department of Pharmaceutical Biology of Kiel University, Stefanie Offt and Olaf Z¨olck, for successfully growing *Leontodon tenuiflorus* far north of its natural range. Additional special thanks for NMR support are due to Gitta Kohlmeyer-Yilmaz from the Institute of Organic Chemistry of Kiel University. Thanks are also due to Dr. Norbert Kilian, Dr. Lars Merkel, and Professor Dr. Roderich Süßmuth (all Berlin) for their efforts in shedding light on the fate of the herbarium of Professor Dr. Ferdinand Bohlmann (1921-1991).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bse.2022.104408.

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Figure captions

Fig. 1. UV HPLC trace recorded at 210 nm. **1**, ixerisoside D; **2**, sonchuside A **3**, 4,6-*O*-dihypocretenoyl-D-glucopyranoside; **4**, linolenic acid; **5**, linolic acid.

Fig. 2. Sesquiterpenoids isolated from *Leontodon tenuiflorus*.

Fig. 3. Key HMBC correlations for compound **3**.

Fig. 4. Phylogenetic position of Leontodon section Asterothrix relative to closely related taxa of the Hypochaeridinae.

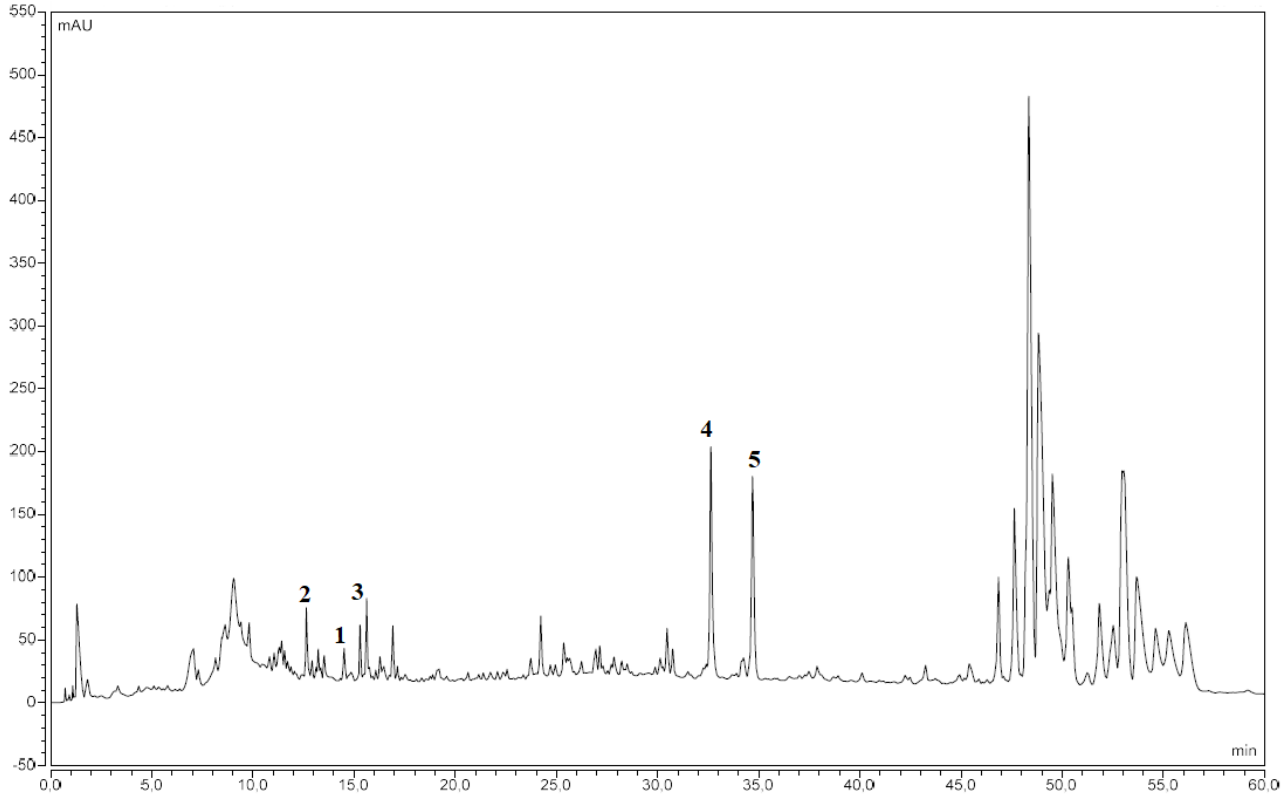
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Fig. 1.

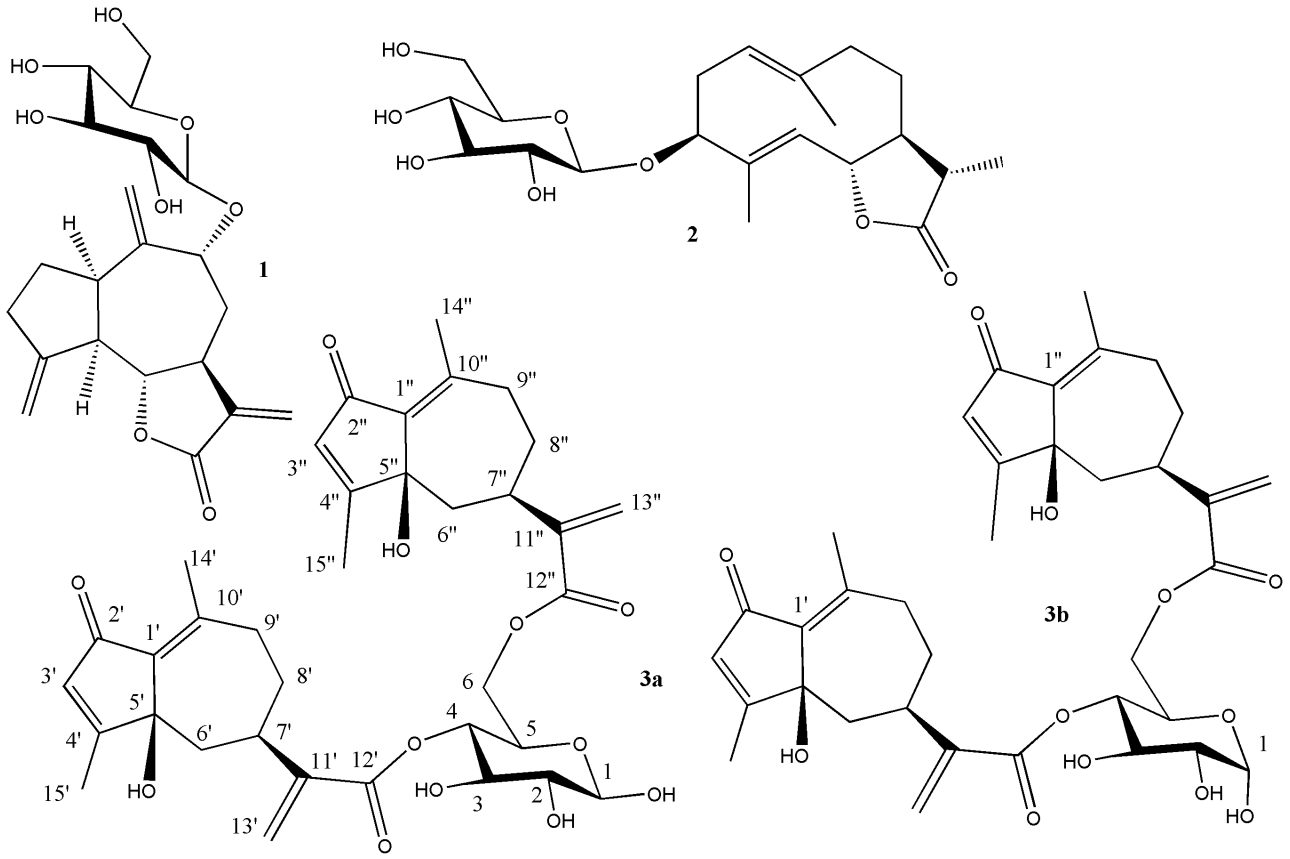


Fig. 2.

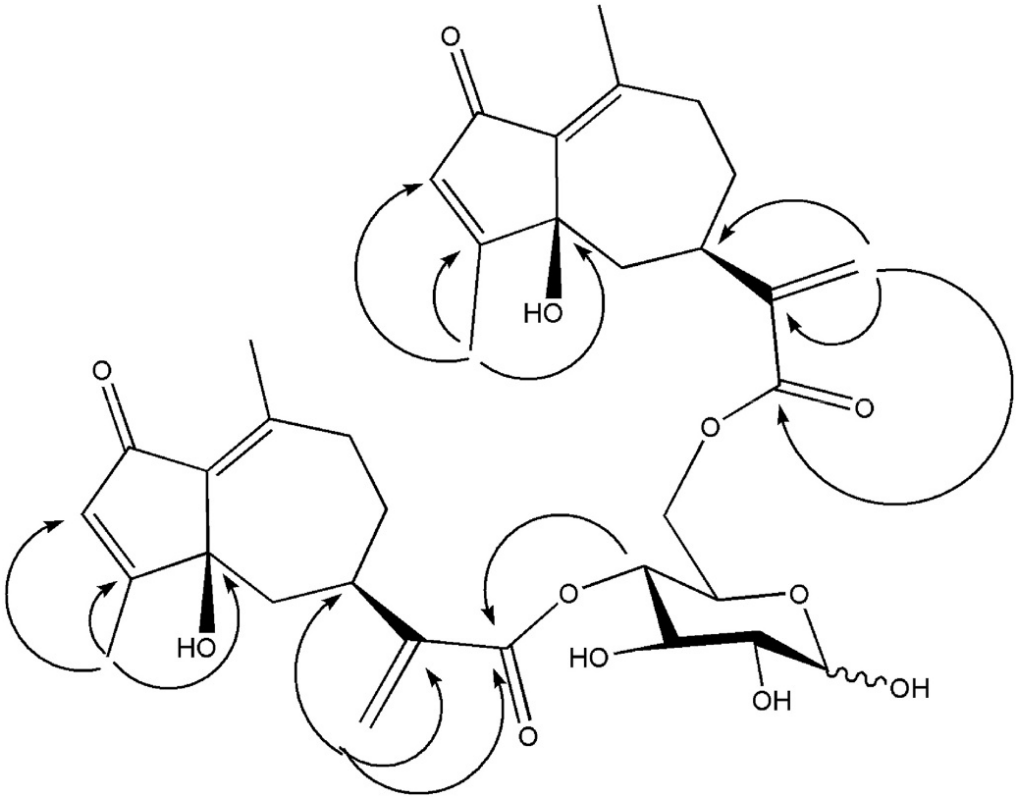
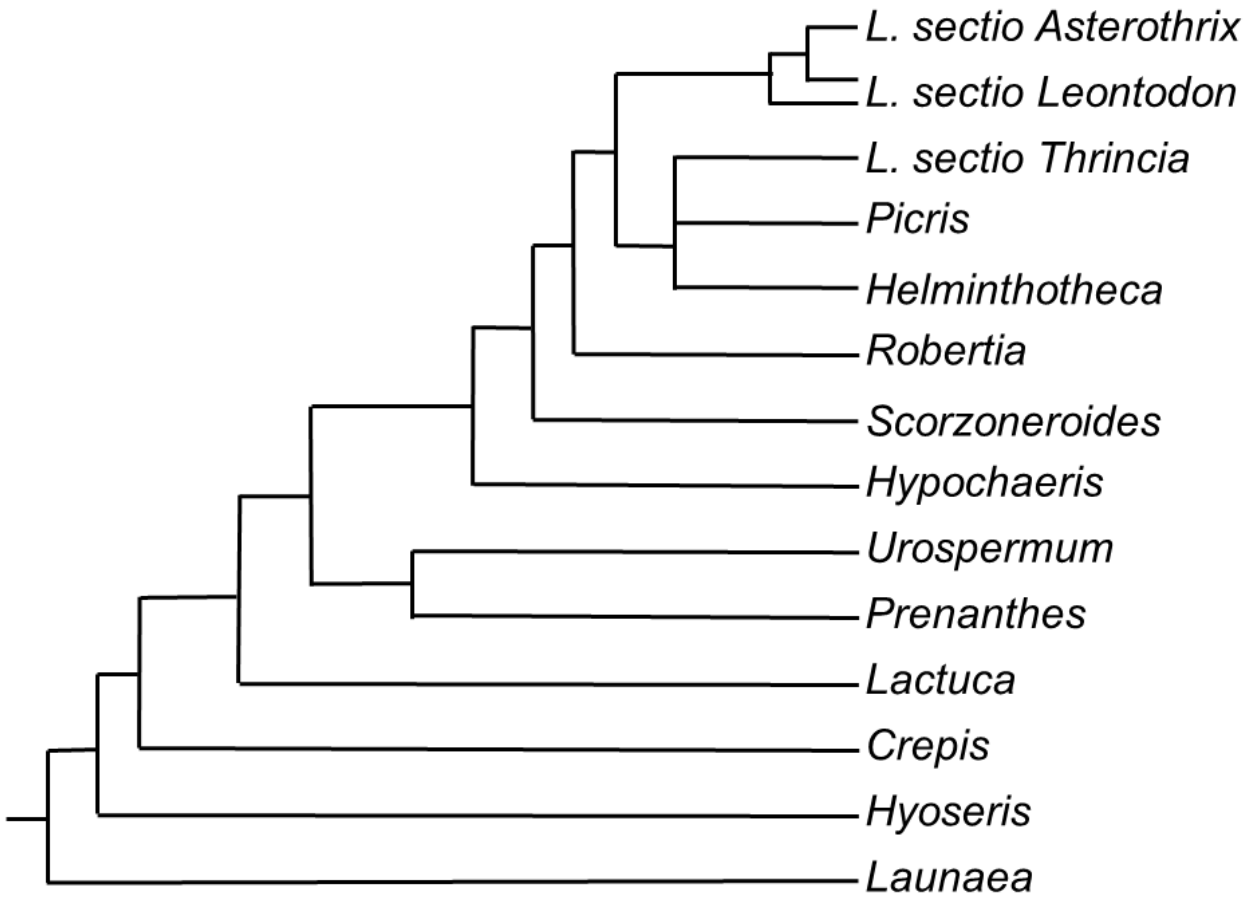


Fig. 3.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Helminthotheca
Robertia
Scorzoneroides
Hypochaeris
Urospermum
Prenanthes
Lactuca
Crepis
Hyoseris
Launaea

Fig. 4.

Table 1. NMR data of compound **3**.

Position	^{1}H NMR	^{13}C NMR	Multiplicity
Glucose (α/β) ratio about 1:1			
1	5.12 0.5H, *d* (4.0)/4.54 0.5H, *d* (7.5)	93.7/98.2	CH
2	3.44 0.5H *dd* (9.0, 4.0)/3.24 0.5H *dd* (9.0, 7.5)	73.6/76.1	CH
3	3.84/3.57 0.5H, *t* (9.0)/ 0.5H, *t* (9.0)	72.3/75.4	CH
4	4.92/4.94 0.5H, *m**/0.5H, *m**	72.9/72.9	CH
5	4.19/3.76 0.5H *m**/0.5H, *m**	68.5/73.3	CH
6	4.16/4.16 2*0.5H *m**/2*0.5H, *m**	63.8/63.8	CH_2
Hypocretenoyl moiety 1 (at O-4)			
1'		135.3	C
2'		198.2	C
3'	5.91 1H, *q* (1,5)	131.7	CH
4'[#]		176.4	C
5'		80.0	C
6'	1.93 2H, *m**	40.8	CH_2
7'	2.53 1H, *m**	37.5	CH
8'[#]	2.16 1H, *m**	28.5	CH_2
	1.70 1H, *m**		
9'[#]	2.97 1H, *m**	36.1	CH_2
	2.23 1H, *m**		
10'[#]		151.6	C
11'[#]		146.9	C
12'		167.5	C
13'	6.21 1H, *br s*	125.0	CH_2
	5.66 1H, *br s*		
14'[#]	2.29 3H, *s*	20.7	CH_3
15'	2.03 3H, *br s*	12.8	CH_3
Hypocretenoyl moiety 2 (at O-6)			
1''		135.4	C
2''		198.1	C
3''	5.88 1H, *q* (1,5)	131.6	CH
4''[#]		176.2	C
5''		80.0	C
6''	1.93 2H, *m**	40.8	CH_2
7''	2.53 1H, *m**	37.5	CH
8''[#]	2.16 1H, *m**	28.1	CH_2
	1.70 1H, *m**		
9''[#]	2.97 1H, *m**	35.9	CH_2
	2.23 1H, *m**		
10''[#]		151.5	C
11''[#]		146.7	C
12''		168.1	C
13''	6.22 1H, *br s*	124.7	CH_2
	5.68 1H, *br s*		
14''[#]	2.29 3H, *s*	20.6	CH_3
15''	2.03 3H, *br s*	12.8	CH_3

Signals were referenced to solvent and solvent residual signals of CD3OD: 3.31 ppm (1H NMR), 49.0 ppm (13C NMR).
[a] Overlapping signals.

[b] Though clearly separated in ^{13}C NMR, this signal might be exchangeable with the respective signal of the other hypocretenolide moiety, because of a missing unambiguous 2D coupling clearly assignable to one of the two hypocretenolide moieties.

Table 2. Occurrence of compounds **1-3** in *Leontodon tenuiflorus* and related *Leontodon* species.

Taxon	Sample	**1**	**2**	**3**
Section Leontodon				
L. hispidus	CZ-20161002A-1	+	n.d.	n.d.
L. hispidus	CZ-20180708C-1	+	n.d.	n.d.
Section Asterothrix				
L. biscutellifolius	CZ-20130519B-4	+	+	+
L. incanus	CZ-99-00379	+	+	n.d.
L. incanus	CZ-L095	+	+	n.d.
L. incanus	CZ-20030524A-1	+	+	+
L. incanus	CZ-20030529A-1	n.d.	n.d.	+
L. incanus	CZ-20030529B-1	n.d.	n.d.	+
L. incanus	CZ-20040523A-1	+	+	n.d.
L. tenuiflorus	CZ-20040521A-1	+	+	+
L. tenuiflorus	CZ-20040521B-1	+	n.d.	+
L. tenuiflorus	CZ-20040522A-1	+	+	+
L. tenuiflorus	CZ-20140529A-1	+	+	+
L. tenuiflorus	CZ-20140608A-1	+	+	+
L. tenuiflorus	CZ-20140713B-1	+	+	+

+ Detected by HPLC-MS-MS; n.d. not detectable by HPLC-MS-MS.