Iridoid Glucosides from *Galium humifusum* Bieb.

Maya Mitova[a], Nedjalka Handjieva[a,*], Mincho Anchev[b] and Simeon Popov[a]

[a] Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria.
Fax: ++3592-700-225. E-mail: IOCHNP@BGCICT.ACAD.BG
[b] Institute of Botany, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

* Author for correspondence and reprint requests

Z. Naturforsch. **54c,** 488–491 (1999); received January 13/March 31, 1999

Galium humifusum, Rubiaceae, Iridoid Glycosides

Two new iridoid glucosides, humifusin A and humifusin B, with a 3-(4-hydroxyphenyl)-propionate ester unit were isolated from *Galium humifusum* together with 11 known iridoid glucosides asperuloside, scandoside, desacetylasperulosidic acid, asperulosidic acid, genipo-sidic acid, desacetylasperuloside, monotropein, V1, V2, V3 and daphylloside. The new iri-doids were identified on the basis of spectral data. ^{13}C NMR data for V1 and V2 iridoids are reported.

Introduction

Galium humifusum (Rubiaceae) is a member of sect. *Galium*, represented with two species in the Bulgarian flora – *G. humifusum* and *G. verum* L. (Anchev, 1989). In continuation of our studies on *Galium* (Mitova *et al.*, 1996a, 1996b; Handjieva *et al.*, 1996), the present paper deals with the study of the iridoid glucosides in *G. humifusum,* known from scattered localities in N. & E. Bulgaria. To our knowledge, no previous phytochemical study of *G. humifusum* has been reported.

Materials and Methods

General experimental procedures

^1H- and ^{13}C-NMR including DEPT and 2D-NMR spectra were recorded on a Brucker DRX 250 MHz spectrometer in CD_3OD, D_2O and $CDCl_3$ and chemical shifts are given in δ (ppm) with TSPA-d_4 and TMS as internal standards. The NOE difference spectra were measured by the use of a standard Bruker software program. Optical rotations were measured on a Perkin Elmer 241 polarimeter. Droplet counter current chromatography was performed on a Büchi 670 apparatus by ascending mode. Aluminium sheets silicagel 60 F_{254} were used for TLC. Reverse phase LPLC was carried out with a Merck Lobar C-18 column size B with H_2O–MeOH mixtures as eluent.

Plant material

Galium humifusum was collected at florescence in the Danube plain (Knezha) (1992 and 1995) and identified from Dr. M. Anchev. The voucher specimens A9283 and A95156 were deposited in the herbarium of the Institute of Botany, Bulgarian Academy of Sciences (SOM).

Extraction and isolation

Dry above-ground parts of *G. humifusum* (163 g) were extracted twice with MeOH (2×1 l) and the concentrated extract (17.2 g) partitioned between $Cl(CH_2)_2Cl$–H_2O (2×300 ml). The aqueous phase was concentrated and treated with charcoal and eluted with H_2O (1.5 l), MeOH–H_2O (5:95, v/v) (1.5 l), MeOH–H_2O (30:70, v/v) (1 l), 50% MeOH (1:1, v/v) (1 l), MeOH–Me_2CO (1:1, v/v) (1 l) and MeOH–$Cl(CH_2)_2Cl$ (1:1, v/v) (1 l). The combined MeOH–Me_2CO (1.3 g) and MeOH–$Cl(CH_2)_2Cl$ (2.2 g) fractions were separated by ascending DCCC with $CHCl_3$–MeOH–H_2O–*n*PrOH (9:12:8:2). Fr. 6–8 (221 mg) were additionally purified using a B size Lobar column and elution with H_2O–MeOH mixtures containing 0.01 M HCOOH to give **8** (fr. 2–5, 15 mg), **5** (fr. 10–19, 30 mg) and **4** (fr. 20–29, 32 mg). Fr. 9 (72 mg), fr. 10–13 (146 mg), fr. 14–22 (311 mg), fr. 56–60 (38 mg), fr. 61–63 (26 mg) contained impure **13**, **7**, **6**, **12** and **11**, respectively. They were additionally purified by Lobar chromatography.

0939–5075/99/0700–0488 $ 06.00

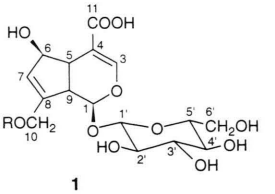
Fr. 23–31 (213 mg) after consecutive purification on a Lobar column B yielded pure **3** (fr. 22–23, 35 mg) and **1** (fr 30, 35 mg). Fr. 42–52 (629 mg) were separated by a Lobar C-18 column with MeOH–H_2O to give pure **9** (fr. 6–7, 316 mg) and **2** (fr. 9, 25 mg). The DCCC stationary phase was collected in fractions of 100 ml. Fr 5 (334 mg) contained pure **10**.

Humifusin A (**1**), 35 mg. [α] −16° (MeOH, c 0.5); UV λ_{max}, nm 226, 280 (MeOH); IR (KBr) cm^{-1}: 3200–3500, 1730, 1700, 1640, 1590, 1505, 1400, 950, 890, 830. ^{1}H-NMR (250 MHz, D_2O): 7.39 (1H, *d*, *J* = 1 Hz, H-3); 7.12 (2H, *d*, *J* = 8.4 Hz, H-5″ and H-9″); 6.81 (2H, *d*, *J* = 8.4 Hz, H-6″ and H-8″); 5.67 (1H, *d*, *J* = 1.2 Hz, H-7); 5.18 (1H, *d*, *J* = 5.6 Hz, H-1); 4.72 (1H, *d*, *J* = 7.9 Hz, H-1′); 4.75 (2H, H_2-10); 4.52 (1H, bs, H-6); 2.85 (4H, *m*, H-5, H-9, H_2-3″); 2.73 (2H, *t*, *J* = 6.1/6.9, H_2-2″). ^{13}C-NMR: Table I.

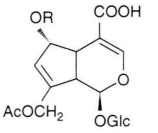
Zemplen reaction of **1.** Humifusin A (12 mg) and 0.1 M NaOMe (5 ml) were refluxed 4 h at 60 °C and the reaction mixture neutralized and concentrated under vacuum (Tanahashi *et al.*, 1996). The reaction product was TLC and HPLC identical with an authentic sample of scandoside.

Humifusin B (**2**). [α] −40° (MeOH, c 0.4); UV λ_{max}, nm 226, 280 (MeOH); IR (KBr) cm^{-1}: 3200–3500, 1729, 1700, 1650, 1620, 1600, 1510, 1255, 905, 825. ^{1}H-NMR (250 MHz, D_2O): 7.35 (1H, *d*, *J* = 1 Hz, H-3); 6.92 (2H, *d*, *J* = 8.4 Hz, H-5″ and H-9″); 6.61 (2H, *d*, *J* = 8.4 Hz, H-6″ and H-8″); 5.35 (1H, *bdd*, *J* = 1/6.6, H-6); 5.21 (1H, bs, H-7); 4.98 (1H, *d*, *J* = 6.9, H-1); 4.82 (1H, *d*, *J* = 8, H-1′); 4.60 (2H, H_2-10), 2.90 (1H, *dt*, *J* = 1/6.6 Hz, H-5); 2.65 (2H, *t*, *J* = 6.5, H_2-3″); 2.50 (3H, *m*, H-9 and H_2-2″) 1.91 (3H, s, Ac). ^{13}C NMR: Table I.

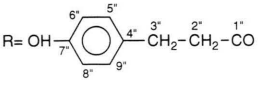
Zemplen reaction of **2**. Humifusin B (9 mg) and 0.1 M NaOMe (4 ml) were refluxed 4 h at 60 °C. The reaction mixture was neutralized and concentrated under vacuum. The reaction product was TLC and HPLC identical with an authentic sample of desacetylasperulosidic acid.

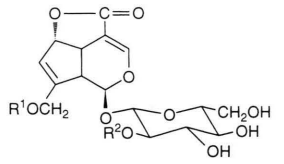


1

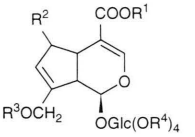


2

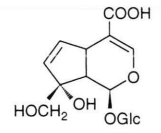




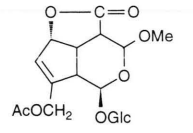
	R^1	R^2
3:	Ac	H
9:	p-$HOC_6H_4(CH_2)_2CO$	H
10:	Ac	p-$HOC_6H_4(CH_2)_2CO$
13:	H	H



	R^1	R^2	R^3	R^4
4:	H	βOH	H	H
5:	H	αOH	H	H
6:	H	αOH	Ac	H
6a:	H	αOAc	Ac	Ac
7:	H	H	H	H
12:	Me	αOH	Ac	H



8



11

ent was asperuloside (**3**) (0.7%), followed by scandoside (**4**) (0.2%) and V1 (**9**) (0.2%) and lower concentrations of desacetylasperulosidic acid (**5**) and V2 (**10**). Minor components were asperulosidic acid (**6**), geniposidic acid (**7**), monotropein (**8**), V3 (**11**), daphylloside (**12**), desacetylasperuloside (**13**), and the new compounds **1** and **2**. Spectral data (El-Nagar and Beal, 1980; Boros and Stermitz, 1990; Chaudhuri *et al.*, 1980) and comparison with authentic samples identified the known iridoid glucosides. Till now, compounds V1 and V2 have been reported only for *G. verum* (Boethe-Horvath *et al.*, 1982a, 1982b). To our knowledge, no ^{13}C NMR data for V1 (**9**) and V2 (**10**) were reported. In Table I are summarized the ^{13}C-NMR data for **9** and **10**.

Compound **1** was obtained as an optically active amorphous powder. The UV spectrum of **1** showed an absorption maximum at 226 nm, typical of a conjugated carbonyl function. The ^{1}H- and ^{13}C-NMR spectra displayed a carboxyl signal at δ_C

Results and Discussion

Two samples collected in different years showed no difference according to HPLC and TLC. Thirteen pure compounds were isolated and identified as the known iridoid glucosides **3-13** in addition to the new compounds **1** and **2**. The major constitu-

171.1, a trisubstituted double bond at δ_H 7.39 (δ_C 152.2) and a acetal signal at δ_H 5.18 (δ_C 97.7). These data and the positive vanillin reaction suggested **1** to be an iridoid. The ^{13}C NMR spectrum of **1** (Table I) contained 25 carbon signals, six of which were readily assigned to a β-glucopyranosyl moiety, ten fitted with a scandoside moiety (**4**) and the remaining nine carbons to a *p*-hydroxyphenylpropionate ester unit. All NMR signals arising for the aglycone and the glucosidic moiety were similar to those of scandoside (**4**) (Chaudhuri *et al.*, 1980) with the exception of those for C-7, C-8 and C-10. No 2D NOESY correlation was observed between H-5 and H-6, which established the *trans*-configuration of H-5 and H-6 and thus, stereochemistry with the β-position of the 6-OH group. Moreover, on Zemplen reaction **1** afforded scandoside. The similar ^{13}C-NMR chemical shifts of C-6 in the spectra of **1** and **4** excluded esterification at this location. The comparison of the ^{1}H- and ^{13}C-NMR chemical shifts for H_2-10, C-8 and C-10 of **1** (δ 4.75; 141.4; 63.1) and **4** (δ 4.20 and 4.28; 146.3; 60.1) determined the site of acylation to be at C-10. The ^{1}H-NMR data for the ester unit was analogous to those reported for the *p*-hydroxypropionyl moiety in compounds **9** and **10** (Böjthe-Horvath *et al.*, 1982a,1982b). The aromatic proton signals coupled in a AA′BB′ system (δ 6.8, *d*, J = 8.4 and δ 7.1, *d*, J = 8.4) and the four signals for aromatic carbons, two of double intensity (C-5″/9″: δ 130.5 and C-6″/8″: δ 116.2) indicated that the ester unit included a para-substituted benzene system. One of the substituents was assumed to be a hydroxyl group taking into account the appearance of a carbon signal at δ 154.7 (C-7″). Additionally in the ^{13}C NMR were observed two methylene

Table I. ^{13}C-NMR chemical shifts of **1**, **2**, **9** (D_2O) and **10** (CD_3OD) and of the model compounds **4**, **6** (CD_3OD) and **6a** ($CDCl_3$).

C-atom	**1**[a]	**4**[b]	**2**[a]	**6**[b] δ_C	**9**[a]	**10**[a]	**6a**[a]
1	97.7 *d*	97.8	98.1 *d*	99.8	93.3 *d*	92.1 *d*	97.6
3	152.2 *d*	153.0	154.4 *d*	153.8	150.4 *d*	149.1 *d*	154.8
4	112.5 *s*	110.0	108.9 *s*	106.6	105.4 *s*	105.4 *s*	106.0
5	46.5 *d*	46.0	41.6 *d*	41.0	36.6 *d*	36.2 *d*	38.4
6	81.3 *d*	81.4	83.0 *d*	73.9	86.8 *d*	85.2 *d*	77.1
7	132.4 *d*	128.9	128.9 *d*	130.5	128.2 *d*	127.9 *d*	128.2
8	141.4 *s*	146.3	143.5 *s*	144.4	142.9 *s*	142.6 *s*	146.7
9	44.4 *d*	44.9	45.6 *d*	46.8	44.1 *d*	43.6 *d*	44.9
10	63.1 *t*	60.1	62.9 *t*	62.3	61.8 *t*	60.9 *t*	61.9
11	171.1 *s*	171.1	171.3 *s*	171.1	173.7 *s*	171.4 *s*	171.4
1′	99.6 *d*	99.2	99.6 *d*	99.1	99.3 *d*	96.3 *d*	100.2
2′	73.5 *d*	73.6	73.5 *d*	73.4	73.4 *d*	73.4 *d*	70.8
3′	76.5[c] *d*	77.1	76.9[c] *d*	77.0	76.3 *d*	74.4 *d*	72.0
4′	70.3 *d*	70.3	70.2 *d*	70.1	70.3 *d*	70.4 *d*	68.2
5′	77.0[c] *d*	76.6	76.4[c] *d*	76.4	77.0 *d*	77.3 *d*	72.3
6′	61.5 *t*	61.5	61.5 *t*	61.5	61.5 *t*	61.5 *t*	61.5
1″	176.2 *s*		175.9 *s*		173.7 *s*	172.9 *s*	
2″	36.4 *t*		36.1 *t*		36.3 *t*	36.1 *t*	
3″	30.3 *t*		30.2 *t*		30.2 *t*	29.6 *t*	
4″	133.0 *s*		132.7 *s*		132.7 *s*	131.9 *s*	
5″, 9″	130.5 *d*		130.3 *d*		130.3 *d*	129.5 *d*	
6″, 8″	116.2 *d*		116.0 *d*		116.1 *d*	115.2 *d*	
7″	154.7 *s*		154.8 *s*		154.9 *s*	155.3 *s*	
$CH_3\underline{CO}$			174.4 *s*	171.2		171.6 *s*	170.5, 170.2, 170.1, 169.9, 169.3, 169.1
$\underline{CH_3}CO$			21.4 *q*	19.4		19.7 *q*	21.0, 20.5 (5Ac)

[a] Multiplicities, determined by DEPT, assignments by HETCOR.
[b] Data from (Chaudhuri *et al.*, 1980); in CD_3OD, 25.2 MHz.
[c] Data interchangeable.

carbon signals at δ 36.4 (C-2″) and δ 30.3 (C-3″) and a carbonyl signal at δ 176.2 (C-1″) attributed to a propionyl moiety. Therefore, the structure of compound **1** was identified as 10-O-[3″-(*p*-hydroxyphenyl)-propionyl]-scandoside, named humifusin A.

Compound **2** also showed UV and NMR spectra for a C-4 substituted iridoid. The ^{13}C-NMR spectrum exhibited 27 signals (Table I), eighteen assigned to an asperulosidic acid moiety (**6**) and nine to a *p*-hydroxyphenylpropionate ester unit. Differences were observed regarding the chemical shifts of the C-6 signal of **2** (δ 83.0) when compared to that of **6** (73.9), which clearly established esterification at C-6. To determine the location of the acetoxy and aromatic ester units the peracetate of asperulosidic acid (**6a**) was prepared. The different shift of the C-6 signal of **2** (δ 83.0) and **6a** (δ 77.1) excluded a location of the acetoxy substituent at C-6. Hence, the acetoxy substituent was located at C-10, while the aromatic one at C-6. The NOESY correlation established the *cis*-configuration of H-5 and H-6 confirming the 6α-OR stereochemistry. Moreover, **2** yielded desacetylasperulosidic acid (**5**) after Zemplen reaction. The unusual strong shielding of the H-7 signal (δ 5.2) and deshielding of the C-6 signal (δ 83.0) were not expected for representatives of the 6α-OR series and were attributed to the presence of the *p*-hydroxyphenylpropionate substituent at C-6. Accordingly, the structure of **2** was identified as 6-O-[3″-(*p*-hydroxyphenyl) propionyl]-asperulosidic acid, named humifusin B.

The isolation from *G. humifusum* and the close related *G. verum* of iridoids with a *p*-hydroxyphenyl-propionate substituent could suggest their role as chemotaxonomic markers for *G. humifusum* and *G. verum* and closely related European members of sect. *Galium*.

Acknowledgements

This work was supported by Grant X-513 of the National Foundation for Scientific Research (Bulgaria).

Anchev M. (1989), *Galium* L. In: Flora R. P. Bulgaricae. **IX** (V. Velchev, ed.). Acad. Sci. Bulg., Sofia, pp. 19–100.

Böjthe-Horvath K., Hetenyi F., Kocsis A., Szabo L., Varga-Balazs M., Mathe I. Jr. and Tetenyi P. (1982a), Iridoid glucosides from *Galium verum*. Phytochemistry **21**, 2917–2919.

Böjthe-Horvath K., Kocsis A., Parkanyi L. and Simon K. (1982b), A new iridoid glucoside from *Galium verum*. First X-ray analysis of a tricyclic iridoid glycoside. Tetrahedron. Lett. **23**, 965–966.

Boros Ch. A. and Stermitz F. R. (1990), Iridoids. An updated review. Part I. J. Nat. Prod. **53**, 1055–1147.

Chaudhuri R. K., Afifi-Yazar F. U. and Sticher O. (1980), ^{13}C NMR spectroscopy of naturally occurring iridoid glucosides and their acylated derivatives. Tetrahedron **36**, 2317–2326.

El-Naggar L. J. and Beal J. L. (1980), Iridoids. Review. J. Nat. Prod. **43**, 649–707.

Handjieva N., Mitova M., Anchev M. and Popov S. (1996), Iridoid glucosides from *Galium album* and *G. lovcense*. Phytochemistry **43**, 625–628.

Mitova M., Handjieva N., Anchev M. and Popov S. (1996a), Iridoid glucosides from four endemics of the *Galium incurvum* group (Rubiaceae). Z. Naturforsch. **51c**, 286–290.

Mitova M., Handjieva N., Spassov St. and Popov S. (1996b), Macedonine, a new non-glucosidic iridoid from *Galium macedonicum*. Phytochemistry **42**, 1227–1229.

Tanahashi T., Shimada A., Kai M., Nagakura N., Inoue K. and Chen Ch. (1996), An Iridoid glucoside from *Jasminum hemsleyi*. J. Nat. Prod. **59**, 798–800.