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Kaempferol-3,4′-di-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside as a new flavonoid from *Iberis amara* L.

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A new flavonol glycoside, kaempferol-3,4′-di-*O*-β-glucopyranoside-7-*O*-α-rhamno-pyranoside, was isolated from the ethanolic extract of the whole fresh plant of *Iberis amara* L., an European plant used in gastrointestinal medicine. The structure was established by a combination of 1D and 2D NMR techniques (COSY, HSQC, HMBC, NOESY) as well as UV, IR and mass spectral data.

1. Introduction

Iberis amara L. is an annual, white to violet blooming plant, reaching up to 40 cm of heights with a strong specific smell, and a bitter cress-like taste. The genus Iberis is growing in Europe, mainly in the mediterranean region (Reichling and Saller 2003). The origin of *Iberis amara* is the controlled cultivation in Germany. The ethanolic extract of Iberis amara totalis is used in a fixed combination with ethanolic extracts of peppermint leaves, matricaria flowers, caraway fruits, balm leaves, celandine herbs, milk thistle fruits, angelica roots and liquorice roots (Iberogast®) in the therapy of gastrointestinal complaints (Rösch et al. 2002; Gundermann et al. 2003).

In the fresh whole plant cucurbitacins with the main components cucurbitacin E and I, and glucosinolates were previously determined (Dalgaard et al. 1977; Nielsen et al. 1977). The flowers contain a wealth of flavonol glycosides of the kaempferol- and quercetin-type: kaempferol-3-*O*-arabinoside-7-*O*-rhamnoside, kaempferol-3-*O*-glucoside-7-*O*-rhamnoside, kaempferol-7-*O*-rhamnoside and quercetin-3-*O*-glucoside-7-*O*-rhamnoside (Kowalewski and Wierzbicka 1971).

As a part of our search for further characteristic flavonoids in this medicinal plant a flavonol glycoside was isolated and the structure was established as kaempferol-3,4′-di-*O*-β-glucopyranoside-7-*O*-α-L-rhamnopyranoside. The experimental data were described for the first time here. In the medicinal used ethanolic extract kaempferol-3,4′-di-*O*-β-glucopyranoside-7-*O*-α-L-rhamnopyranoside is a major compound of the flavonoid pattern (Kroll and Cordes 2006).

2. Investigations, results and discussion

For isolation the residue of the ethanolic extract of the whole fresh plant of *Iberis amara* was treated with methanol/water. After purification with solid phase extraction on an aminopropyl phase the concentrate was separated by preparative HPLC with C18 and aminopropyl material using acetonitrile and water-mixture gradients to afford the major compound of the flavonoid pattern. The isolated compound was obtained as a light beige to yellow powder, m.p. 200–215 °C (dec.). It was characterized as a flavonoid on the basis of its UV absorption (λ_{max} 267 nm, 320 nm and 344 nm).

Based on ESI-MS data (m/z 757 $[M + H]^+$) the compound had a molecular formula of $C_{33}H_{40}O_{20}$. The IR spectrum showed diagnostic absorption bands at 3408 (ν O–H), 2900 (ν CH_3), 1668 and 1600 (ν C=O, C=C) cm^{-1} which imply the existence of keto groups and aromatic systems. Fig. a shows the total ion spectrum of the compound. It exhibited a protonated molecular ion $[M + H]^+$ at m/z 757 and fragments $[M + H\text{-}162]^+$, $[M + H - 162 - 146]^+$ and $[M + H - 2 \times 162 - 146]^+$ at

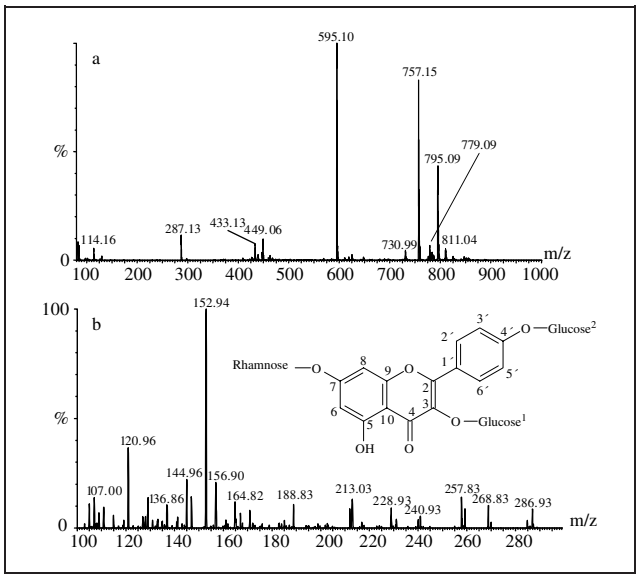

Fig.: MS spectrum (a) and MS/MS spectrum (b) of the parent ion m/z 287 of kaempferol-3,4′-di-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside

Table: ^{1}H and ^{13}C NMR spectral data for kaempferol-3,4′-di-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside (DMSO-d_6)

Position	Aglycone		Position	Sugar moieties					
				Glucose[1]		Glucose[2]		Rhamnose	
	δ_H[a]	δ_C[b]		δ_H[a]	δ_C[b]	δ_H[a]	δ_C[b]	δ_H[a]	δ_C[b]
2	–	156.19 C	1	5.49 d (7.5)	100.78 CH	5.02 d (7.3)	99.98 CH	5.56 d (1.2)	98.44 CH
3	–	134.07 C	2	3.19 m	74.34 CH	3.28 m	73.27 CH	3.84 brs	69.85 CH
4	–	177.81 C	3	3.22 m	76.46 CH	3.29 m	76.55 CH	3.63 m	70.28 CH
5	–	160.94 C	4	3.08 m	69.98 CH	3.19 m	69.66 CH	3.29 m	71.65 CH
6	6.46 d (2.2)	99.51 CH	5	3.09 m	77.68 CH	3.40 m	77.12 CH	3.44 m	70.13 CH
7	–	161.75 C	6	3.58 brdd	60.93 CH_2	3.70 brdd	60.68 CH_2	1.11 d (6.1)	17.96 CH_3
8	6.85 d (2.2)	94.67 CH		3.33 m		3.48 m			
9	–	156.13 C							
10	–	105.83 C	2-OH	5.36 d (4.7)		5.40 d (4.8)		5.17 d (4.1)	
1′	–	123.62 C	3-OH	5.09 d (4.7)		5.13 brs		4.81 brs	
2′	8.15 d (9.1)	130.77 CH	4-OH	4.98 d (4.4)		5.07 d (5.7)		4.94 d (5.4)	
3′	7.16 d (9.1)	115.89 CH	6-OH	4.34 t (5.7)		4.61 t (5.7)			
4′	–	159.40 C							
5′	7.16 d (9.1)	115.89 CH							
6′	8.15 d (9.1)	130.77 CH							
5-OH	12.54 brs								

[a] J values (Hz) given in parentheses; [b] C multiplicities determined from DEPT
Assignments confirmed by 2D NMR ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY spectra

m/z 595, 449 and 287, respectively. The ions m/z 795 and 779 correspond to the molecular potassium and sodium adducts $[M + K]^+$ and $[M + Na]^+$. The differences of 162 u and 146 u are characteristic for the cleavage of O-glycosides. Therefore, these data indicate the loss of three monosaccharide moieties in the form of desoxyhexosyl [−146] and hexosyl [−162] units. As a consequence, the fragment m/z 287 which was also observed in the LC-ESI-MS can be assigned to the aglycone moiety. Nevertheless, on the basis of LC-ESI-MS data alone it was not possible to determine if the aglycone was kaempferol or luteolin, two flavonoids which have two hydroxy groups either on the ring A or B and, in addition, one or no hydroxy group in position C-3, respectively. To obtain more information on the distribution of the substituents of the A- and B-ring, LC-ESI-MS/MS was carried out by choosing the ion $[M + H - 2 \times 162 - 146]^+$ as parent ion (Fig. b). In this case, a characteristic retro-Diels-Alder fragmentation of the aglycone moiety gave an ion at m/z 153. This fragment indicates an A-ring having two hydroxy groups. The ions at m/z 121 and 137, which are specific for the B-ring, showed different intensities with ion m/z 121 being more pronounced than m/z 137. This distribution is typical for flavonols having a hydroxy group on the ring B and one in position C-3, as it is the case in kaempferol (Hostettmann et al. 1997).

The ^{1}H and ^{13}C NMR spectra of the compound (see Table) showed resonances for a flavonol and three sugar moieties. Careful analysis of the signals and comparison of the data with those in literature (Agrawal and Bansal 1989) led to kaempferol, two β-linked glucopyranosides ($J_{H-1/H-2} = 7.3$ and 7.5 Hz) and one α-linked rhamnopyranoside ($J_{H-1/H-2} = 1.2$ Hz) (Markham and Geiger 1986). The linkages of all moieties were obtained from HMBC correlations of the anomeric protons of the three sugar moieties to carbon atoms of the aglycone. Key correlations included: H-1 (δ_H 5.49) of glucose[1] to C-3 (δ_C 134.07), H-1 (δ_H 5.02) of glucose[2] to C-4′ (δ_C 159.40) and H-1 (δ_H 5.56) of rhamnose to C-7 (δ_C 161.75). Therefore, the isolated compound is kaempferol-3,4′-di-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside.

3. Experimental

3.1. Materials and methods

Whole fresh plants of *Iberis amara* L., Brassicaceae, were harvested and classified by Steigerwald Arzneimittelwerk GmbH, Darmstadt in 2000. Voucher specimens (batch 00-0015, 00-0016) were kept at Steigerwald Arzneimittelwerk GmbH.

The LC-MS/MS system comprised an Quattro Ultima triple quadrupole mass-spectrometer fitted with an ionspray interface (Micromass, Eschborn, Germany) and coupled to an Alliance 2695 LC system (Waters, Eschborn, Germany). UV spectra in the range of 210 to 400 nm were monitored by an in-line arranged photodiode array detector. After UV detector the solvent flow was splitted at a ratio of 3 : 1 (waste/MS). Analyses were performed with electrospray ionisation (ESI) in the positive mode. The extract was separated on a polar C18 reversed phase column (Aqua 125A C18 250 × 4.6 mm, 5 µm, Phenomenex, Aschaffenburg, Germany) with a guard column (4 × 2 mm) packed with the same reversed phase material (Phenomenex, Aschaffenburg, Germany) at 25 °C. The following pump program was used: 90% solvent A (water pH 2.65, adjusted with formic acid) for 1 min, then decreasing solvent A to 45% within 50 min and further to 0% within 10 min, followed by a cleaning and conditioning step. Solvent B consisted of a mixture of water (pH 2.86, adjusted with formic acid) and acetonitrile (50 : 50, v/v). The flow rate was 1.0 ml/min. The ionspray source was operated at 400 °C, 3.5 kV capillary voltage and 50 V cone voltage. The collision energy for MS/MS ranged from 10 to 40 eV with argon CAD gas. Total and daughter ion experiments were performed. IR spectra were measured in a KBr pellet using a Nicolet Impact 400 IR spectrometer. NMR spectra were recorded on a Bruker Avance 500 (500 MHz) spectrometer. ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY spectra were obtained with the usual pulse sequences. All NMR spectra were performed in DMSO-d_6. Chemical shifts are given in δ values (ppm) relative to the solvent signal at δ_H 2.50 or δ_C 39.50.

3.2. Extraction and isolation of kaempferol-3,4′-di-O-β-glucopyranoside-7-O-α-rhamnopyranoside

Whole fresh plants of *Iberis amara* L. were extracted with 30 % EtOH (1 : 2) in the usual production process. 100 l of the ethanolic extract was evaporated in vacuum at 45 °C to dryness (1900 g). The residue (50 g) was recrystallized in methanol/water (1 : 1). The precipitate obtained was purified with solid phase extraction on an aminopropyl phase and the concentrate was separated by preparative HPLC (UV 276 nm) using C18 and aminopropyl material. Acetonitrile and water-mixture were used as solvent. The fractions were collected and evaporated in vacuum to dryness. The residue (800 mg) was dissolved in methanol (2 ml) and diluted with t-butylmethyl ether (200 ml). Repeated crystallization from methanol/t-butylmethyl ether gave bright yellow crystals. Evaporation to dryness (650 mg) in vacuum at max. 45 °C led to kaempferol-3,4′-di-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside.

3.3. Characterization of kaempferol-3,4′-di-O-β-glucopyranoside-7-O-α-rhamnopyranoside

Light beige to yellow powder; m.p. 200–215 °C (dec.); UV: λ_{max} (MeOH) nm: 267, 320, 344, IR (KBr): ν_{max} cm^{-1}: 3408, 2900, 1668, 1600, 1085, δ_{max} cm^{-1}: 1450–1300, 800–700; ESI-MS m/z (795) $[M + K]^+$, m/z (779) $[M + Na]^+$, m/z (757) $[M + H]^+$, m/z (595) $[M + H - 162]^+$, m/z (449) $[M + H - 162 - 146]^+$, m/z (287) $[M + H - 2 \times 162 - 146]^+$; ^1H and ^{13}C NMR: see Table.

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