HETEROCYCLES, Vol. 65, No. 7, 2005, pp. 1655 - 1661
Received, 20th January, 2005, Accepted, 25th April, 2005, Published online, 28th April, 2005

FLAVONE *C*-GLYCOSIDES FROM *ISATIS TINCTORIA* LEAVES

Yongxian Cheng, [a] Bernd Schneider, [b] Christine Oberthür, [a,c] Heidemarie Graf, [a] Sven Adler, [a] and Matthias Hamburger [a,c,*]

[a]Institute of Pharmacy, Friedrich-Schiller-University Jena, Semmelweisstrasse 10, D-07743 Jena, Germany, [b]Research Group Biosynthesis / NMR, Max Planck Institute for Chemical Ecology, Beutenberg Campus, Hans-Knöll-Strasse 8, D-07745 Jena, Germany, [c]Institute of Pharmaceutical Biology, Pharmacenter, University of Basel, Klingelbergstrasse 56, CH-4056 Basel, Switzerland, E-mail address: matthias.hamburger@unibas.ch

Abstract – Two new flavone *C*-glycosides, chrysoeriol 6-*C*-β-D-glucopyranosyl-(1→3)-β-glucopyranoside (**1**) and apigenin 6-*C*-β-D-glucopyranosyl-(1→3)-β-glucopyranoside (**2**) were isolated from a methanolic extract of *Isatis tinctoria* leaves and identified on the basis of spectral analysis and hydrolysis. Two known flavone *C*-glycosides, isoscoparin (**3**) and isovitexin (**4**), were also identified.

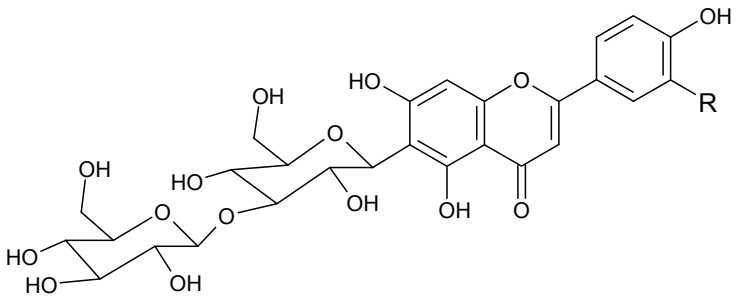
INTRODUCTION

The woad plant *(Isatis tinctoria*, Brassicaceae) is an ancient indigo dye and medicial plant which has been used and cultivated in temperate climate zones of Central Europe since antiquity.[1,2] A related species, *I. indigotica* [3], is grown in East Asia and used as a medicinal plant in Chinese medicine.[4,5] We recently confirmed the anti-inflammatory potential of *Isatis tinctoria* in a broad-based pharmacological profiling,[6] and identified the alkaloid tryptanthrin as the cyclooxygenase-2 (COX-2) inhibitory principle in woad leaves.[7] The compound was found to be a potent inhibitor of COX-2 and 5-lipoxygenase (5-LOX) catalyzed eicosanoid synthesis.[8] When we discovered that tryptanthrin was not present in the live plant but formed only after harvest during the drying process,[9] we embarked on a phytochemical investigation on putative precursor molecules. Among these, we characterized the indigo precursors[10] and studied their post-harvest degradation.[11] These analyses revealed that the metabolite profiles in woad were subjected to profound changes in the post-harvest period. Since earlier phytochemical studies were mostly with dried plant material, a comprehensive characterization of the metabolite profile in fresh plant was needed. As a

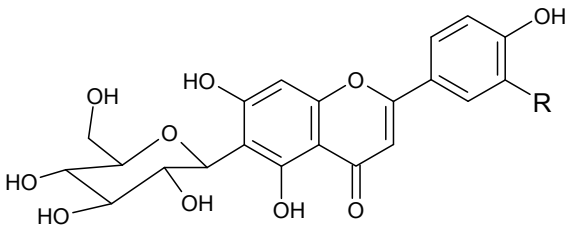
part of this study, we analyzed the flavonoid pattern in leaves, and we here report on the isolation and structure elucidation of known and new flavone *C*-glycosides.

RESULTS AND DISCUSSION

Compounds (**1**-**4**) were isolated from a methanolic leaf extract by a combination of normal phase chromatography on silica gel, gel chromatoghraphy on Sephadex LH-20, and by a final separation of the two pairs of flavone monoglycosides (**3** and **4**) and diglycosides (**1** and **2**), by semi-preparative HPLC on a C-18 column.



1 R=OCH_3
2 R=H



3 R=OCH_3
4 R=H

The molecular formula of **1** was established as $C_{28}H_{32}O_{16}$ on the basis of a HR ESIMS spectrum. The UV spectrum with absorption maxima at 243, 271 and 347 nm was characteristic of a flavonoid. The ^{1}H NMR spectrum of compound (**1**) (**Table 1**) in MeOH-d_4 exhibited signals of an aromatic ABX spin system (δ 7.52, 7.47, 6.94), two singlets of another aromatic moiety (δ 6.51; δ 6.65), an aromatic methoxyl singlet (δ 3.96), and signals attributable to two carbohydrate moieties, such as two anomeric protons (δ 4.61; δ 4.94). The ^{13}C NMR spectrum (**Table 1**) showed resonances of 28 carbons, which, by means of a DEPT-135 spectrum (MeOH-d_4), were assigned to one methoxyl (δ 57.0), two hydroxymethylene groups of the sugar units (δ 62.9, δ 63.1), 15 methine and ten quaternary carbon atoms. The data suggested a flavonoid diglycoside with both sugar units being hexoses. ^{1}H-^{1}H-COSY and ^{1}H-^{13}C heteronuclear correlation experiments (HMBC, HMQC) in MeOH-d_4 indicated a 3'-methoxy-4'-hydroxyphenyl-5,7-dihydroxyflavone skeleton of the aglycone moiety. Thus, the aglycone of (**1**) was shown to be chrysoeriol.[12] Confirmatory measurements in DMSO-d_6 revealed hydroxyl protons at δ 9.96 (OH-4'), δ 10.72 (OH-7) and δ 13.64 (OH-5), which were assigned by HMBC connectivities. The carbohydrate moiety was a disaccharide attached to the only remaining position at C-6. The *C*-glycosidic structure was established from the chemical shifts of C-6 (δ 109.1 in MeOH-d_4) and C-1'' (δ 75.1) [13] and by HMBC correlations of H-1'' (δ 4.94 in MeOH-d_4) with C-5 (δ 162.3) and C-7 (δ 165.5) and with C-6. Attachment of the second hexosyl moiety at C-3'' was indicated by a HMBC correlation of H-1''' with C-3''(δ 90.3 in MeOH-d_4), and by a missing hydroxyl proton signal of OH-3'' in the spectrum measured

in DMSO-d_6. The large coupling constants $J_{H\text{-}1'' - H\text{-}2''}$ = 9.9 Hz and $J_{H\text{-}1''' - H\text{-}2'''}$ = 7.8 Hz indicated β-configuration of both sugar moieties. Axial orientations of the remaining sugar protons H-2'' through H-5'', and H-2''' through H-5''' were deduced from their large *J* values (**Table 1**) and indicated the presence of two glucosyl moieties. Finally, the multiplicities of the carbohydrate hydroxyl protons (two triplets at δ 4.56 and 4.54 and five doublets, see Table 1) observed in DMSO-d_6, together with their HMBC connectivities with the corresponding carbons, confirmed the carbohydrate part as laminaribiose. Acid hydrolysis of **1** and purification of the monosaccharide gave a product with positive optical rotation in water. The terminal glucose thus belonged to the D-series, and compound (**1**) was, therefore, chrysoeriol–6–*C*–β-D-glucopyranosyl-(1 → 3)-β-glucopyranoside.

Compound (**2**) was initially observed in the NMR spectrum of **1** as a minor component (6 %), and subsequently purified and analysed by NMR and MS spectral techniques. The ^{1}H NMR spectrum (MeOH-d_4) closely resembled that of compound (**1**) with exeption of the signal pattern of the lateral phenyl ring. Two doublets (δ 7.86 and 6.93) integrating for two protons each appeared instead of the ABX system, indicated a *p*-substituted ring. Although only 70 µg of compound (**2**) was available, ^{1}H-^{1}H COSY and ^{1}H-^{13}C heterocorrelation experiments (HMBC, HMQC) were recorded using a 3 mm cryoprobe, which enabled complete assignment of all ^{1}H and ^{13}C resonances. The *C*-glucosidic character of (**2**) again was established from the high-field resonance of C-1'' (δ 75.0) and a HMBC cross signal between H-1'' (δ 4.92) with C-6 (δ 109.3). Another HMBC correlation of H-1''' (δ 4.62) with C-3'' (δ 90.2), resonating at relatively low field, indicated the link between the two glucose units and confirmed the disaccharide as laminaribiose. Thus compound (**2**) was determined as apigenin 6-*C*-β-glucopyranosyl-(1 → 3)-β-glucopyranoside. The two other flavone *C*-glycosides were identified as isoscoparin (**3**)[12] and isovitexin (**4**)[14] respectively.

Two new flavone-*C*-diglycosides (**1**) and (**2**) with the rare 1→3 interglycosidic linkage and the two corresponding monoglycosides (**3**) and (**4**), have been identified in freshly harvested leaves of *Isatis tinctoria*. This has been the first systematic investigation of flavonoid pattern in this formerly important commercial dye plant which we currently investigate for its pronounced anti-inflammatory properties in vivo and in humans.[15,16] The flavonoid pattern in Brassicaceae has been investigated. *O*-Glycosides of flavonols kaempferol, quercetin and isorhamnetin appear predominantly. *O*- and *C*-glycosides occur occasionally, and rarely flavanones and flavanonols and chalcones.[17-19] Isoscoparin has been previously identified in seeds of *Isatis tinctoria*, but the leaves were not investigated at that time.[20] In the workup process of the leaves and by HPLC-PDA analysis, no further flavonoid-like compounds were detected. It thus appears that flavone *C*-glycosides (**1**-**4**) are the characteristic flavonoids in *Isatis tinctoria* leaves.

Table 1. ^{1}H (500 MHz) and ^{13}C (100 MHz) NMR Spectral Data of **1** and **2**

	1		**1**		**2**	
pos	δ_C [a]	δ_H (mult.; J_{HH} in Hz) [a]	δ_C [b]	δ_H (mult.; J_{HH} in Hz) [b]	δ_C	δ_H (mult.; J_{HH} in Hz) [a]
2	166.1		163.4		163.0	
3	104.5	6.65 s	103.2	6.90 s	103.9	6.61 s
4	184.1		182.0		183.7	
5	162.3		160.8		162.5	
5-OH				13.64 s		
6	109.1		108.4		109.3	
7	165.5		163.3		165.3	
7-OH				10.72 brs		
8	95.4	6.51 s	93.7	6.55 s	95.3	6.50 s
9	158.9		156.3		159.1	
10	105.1		103.2		105.3	
1’	123.7		121.4		123.8	
2’	110.8	7.47 d (2.1)	110.2	7.52 brs	129.4	7.86 d (8.7)
3’	149.6		148.0		117.2	6.93 d (8.7)
4’	152.3		150.7		166.1	
4’-OH				9.96 s		
5’	117.0	6.94 d (8.4)	115.8	6.93 d (8.3)	117.2	6.93 d (8.7)
6’	122.0	7.52 dd (8.4, 2.1)	120.4	7.56 dd (8.3, 2.0)	129.4	7.86 d (8.7)
1’’	75.1	4.94 d (9.9)	72.5	4.66 d (10.0)	75.0	4.92 d (9.9)
2’’	72.0	4.42 dd (9.9, 9.5)	68.6	4.32 m	71.9	4.43 dd (9.9, 9.5)
2’’-OH				4.82 d (4.1)		
3’’	90.3	3.63 dd (9.5, 8.8)	90.6	3.36 m	90.2	3.64 m
4’’	70.7	3.57 dd (8.8, 8.8)	69.0	3.22 m	70.5	3.56 dd (8.8, 8.8)
4’’-OH				4.67 brs		
5’’	82.9	3.44 m	81.4	3.22 m	82.9	3.44 m
6’’a	63.1	3.73 dd (12.0, 5.4)	61.3	3.41 m	62.9	3.73 dd (12.0, 5.4)
6’’b		3.89 dd (12.0, 2.1)		3.69 m		3.89 dd (12.0, 2.1)
6’’-OH				4.54 t (6.0)		
1’’’	105.4	4.61 d (7.8)	104.1	4.34 d	105.5	4.62 d (7.8)
2’’’	75.8	3.30 [c]	73.8	3.02 m	75.8	3.29 m
2’’’-OH				5.04 d (3.2)		
3’’’	78.2	3.39 dd (9.0, 9.0)	76.0	3.17 m	78.0	3.38 dd (9.0, 9.0)
3’’’-OH				5.00 d (4.6)		
4’’’	71.9	3.29 [c]	70.1	3.04 m	71.7	3.28 m
4’’’-OH				4.98 d (5.5)		
5’’’	78.4	3.42 m	76.9	3.21 m	78.4	3.34 m
6’’’a	62.9	3.64 dd (12.1, 5.3)	61.1	3.41 m	62.7	3.65 m
6’’’b		3.90 dd (12.1, 2.1)		3.69 m		3.90 dd (12.1, 2.1)
6’’’-OH				4.56 t (6.0)		
OMe	57.0	3.96 s	56.0	3.88 s		

[a] recorded in MeOH- d_4; [b] recorded in DMSO-$d_{6.,}$ [c] overlapped signals.

No flavonoids could be detected in a chromatographic analysis of the roots. In contrast, the roots of the closely related *I. indigotica*, an important Chinese medicinal plant[4,5] appears to contain *O*- and *C*-glycosides linarin and homovitexin.[21] Differences in flavonoid pattern may thus serve as marker for the detection of adulterations and confusions between *I. indigofera*, *I. tinctoria* and other closely related species, but a detailed analysis of different plant parts and genotypes is needed to confirm the diagnostic value of flavonoid pattern.

EXPERIMENTAL

General Experimental Procedures. Solvents and chemicals were of analytical or HPLC grade unless indicated otherwise. Silica gel (40-63 μm) for column chromatography was from Macherey & Nagel (Düren, Germany). Optical rotations were determined on a Polartronic E polarimeter (Schmidt and Haensch, Berlin) equipped with a 5 cm microcell. UV spectra were recorded on a Beckman DU640 spectrophotometer. ^{1}H NMR, ^{1}H,^{1}H COSY, HMBC, and HMQC spectra were recorded with a Bruker AVANCE DRX 500 spectrometer at 500.13 MHz using a 5 mm CryoProbe^{TM} or a 3 mm CryoProbe^{TM}; ^{13}C NMR and DEPT spectra were measured using a Bruker AVANCE 400 spectrometer at 400.13 MHz. ^{13}C chemical shifts of compound (**2**) were obtained from ^{1}H-^{13}C heterocorrelated 2D spectra. Samples were measured at 300 K in DMSO-d_6 or MeOH-d_4 and TMS was used as an internal standard. LC-ESIMS spectra were determined in the positive ion mode on a PE Biosystems API 165 single quadrupole instrument. HRESIMS (positive ion mode) spectra were recorded on a Thermo Finnigan MAT 95 XL mass spectrometer.

Plant Material, Extraction and Isolation. Leaves used for isolation were harvested in August 2001 from first-year plants of the defined *Isatis tinctoria* strain "Jenaer Waid", cultivated on experimental plots of the Agricultural Research Station of Thuringia (TLL), Dornburg, Germany. Leaves were frozen (-32° C) immediately after collection. Frozen leaves were extracted in portions of 150 g in a *Waring* blender (Waring, New Hartford, USA) for 2 min at rt with methanol (450 mL). The plant material was filtered off, and the solvent evaporated under reduced pressure to afford 5 g of extract. A total of 50.2 g of extract was prepared. An aliquot of the extract (16.8 g) was dissolved in chloroform/methanol/water (65:30:5; 600 mL), and silica gel (100 g) was added. The solvent was evaporated under reduced pressure and the residue lyophylized. The adsorbed extract was subjected to column chromatography on silica gel (72 x 8.2 cm i.d.) using a step gradient $CHCl_3$-MeOH-H_2O (90:10:0→80:18:2→60:35:5). A total of 10 fractions (Fr. 1-10) were collected on the basis of the TLC pattern. Two flavonoid containing fractions (Fr. 5, 1.45 g and Fr. 7, 2.40g) were obtained. Fraction 7 containg the diglycosides was subjected twice to chromatography on Sephadex LH-20 (eluent methanol) to afford a mixture of **1** and **2** (67 mg). Compounds (**1**) (15.2 mg) and (**2**) (0.1 mg) were separated by semi-preparative HPLC on a LiChrosorb RP-18 column (7 μm, 25 x 2.5 cm i.d.; Merck, Darmstadt, Germany) using 17% acetonitrile in water as mobile phase. Gel chromatography on Sephadex LH-20 (eluent methanol) of fraction 5 afforded a mixture (55 mg) of two flavonoids which was separated by semi-preparative HPLC on a LiChrosorb RP-18 column (7 μm, 25 x 2.5 cm i.d.; Merck) using 17% aqueous acetonitrile as mobile phase to afford **3** (11.2 mg) and **4** (1.5 mg).

C*hrysoeriol 6-C-β-D-glucopyranosyl-(1→3)-β-glucopyranoside* (**1**). Yellow amorphous powder; $[\alpha]_D$ -29.5^{o} (c 0.88, in DMSO); UV (MeOH) λmax (log ε) 347 (4.28), 271 (4.15), 243 (4.15) nm; ^{1}H-NMR

(500 MHz, DMSO-d_6 and MeOH-d_4), see **Table 1**; ^{13}C-NMR (125 MHz, DMSO-d_6 and MeOH-d_4), see Table 1; ESI-MS (pos.), *m/z*: 625 $[M+H]^+$ (100); HR-ESI-MS (pos.), *m/z*: 647.1586 $[M+Na]^+$, calcd for $C_{28}H_{32}O_{16}Na$, 647.1588.

Apigenin 6-C-β-glucopyranosyl-(1→3)-β-glucopyranoside (**2**). Yellow film; ^{1}H-NMR (500 MHz, MeOH-d_4), see **Table 1**; ^{13}C-NMR (125 MHz, MeOH-d_4), see Table 2; ESI-MS (pos.), *m/z*: 617 $[M+Na]^+$ (100); HR-ESI-MS (pos.), *m/z*: 617.1548 $[M+Na]^+$, calcd for $C_{27}H_{30}O_{15}Na$, 617.1482.

Acid hydrolysis of 1. A solution of **1** (7 mg) in 1 mol/L HCl (2 mL) was kept at 90 ^{O}C for 3 h. The reaction mixture was neutralized with 1 mol/L NaOH and then passed through a RP-18 cartridge eluted with H_2O and then MeOH. The MeOH fraction showed a spot on TLC ($CHCl_3$-MeOH-H_2O (60:35:5); Rf = 0.70) which had the same Rf as compound (**3**). The sugar fraction was lyophilized and extracted by solid phase extraction on a RP-18 cartridge (40-63 µm; 5.0 x 0.5 cm i.d.) eluted with water to afford a mixture of monosaccharide and sodium chloride. The eluate was lyophilized and the sugar purified by vacuum liquid chromatography on a silica gel column (40-64 µm; 5.0 x 0.5 cm i.d.) using CH_2Cl_2-MeOH (2 : 1) as mobile phase. The monosaccharide had the same chromatographic mobility on TLC as pure glucose ($CHCl_3$-MeOH-H_2O (60:35:5); Rf = 0.35; staining with 10% sulphuric acid in ethanol). D-glucose was confirmed by ESI-MS (pos.), *m/z*: 203 $[M+Na]^+$ (100), 185 $[M+Na-H_2O]^+$(6), and optical rotation in water (positive).

ACKNOWLEDGEMENTS

We are grateful to Dr. A. Vetter and S. Biertümpfel, Agricultural Research Station of Thuringia (TLL), Dornburg, for the provision of plant material from the experimental plots for woad cultivation. We are indebted to Mr. Johannes Wange, Institute of Pharmacy, Friedrich-Schiller University Jena, for measuring optical rotation, and Dr. Wolfgang Poppitz, Institute of Inorganic Chemistry, Friedrich-Schiller University Jena, for recording HRESIMS.

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