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C-Glucosylflavonoid biosynthesis from 2-hydroxynaringenin by *Desmodium uncinatum* (Jacq.) (Fabaceae)

Mary L. Hamilton, John C. Caulfield, John A. Pickett, Antony M. Hooper *

Biological Chemistry Department, Centre for Sustainable Pest and Disease Management, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK

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ABSTRACT

[2′,3′,5′,6′-$^2H_4$]-2-Hydroxynaringenin is synthesised and incubated with commercially available UDP-glucose and the crude protein extract from *Desmoduim uncinatum* leaves. The organic extract produces isotopically labelled [2′,3′,5′,6′-$^2H_4$]-vitexin and [2′,3′,5′,6′-$^2H_4$]-isovitexin. Repeating the experiment with denatured protein or replacing the 2-hydroxynaringenin with [2′,3′,5′,6′-$^2H_4$]-apigenin or [2′,3′,5′,6′-$^2H_4$]-naringenin results in no observable incorporation. 2-Hydroxynaringenin is therefore the substrate for *C*-glucosylflavonoid biosynthesis in *D. uncinatum*.

Desmodium uncinatum (Jacq.) is used as an intercrop in subsistence farming of maize (*Zea mays*) in East Africa where it has demonstrated effectiveness in the suppression of parasitism by the parasitic plant *Striga hermonthica* (Del.) Benth., a weed that can devastate crops in the region.[1–4] The effect was shown to be allelopathic[5] and during investigations into the mechanism, we have discovered *C*-glycosylflavonoids, in particular the di-*C*-glycosylflavonoid, 6-*C*-α-L-arabinopyranosyl-8-*C*-β-D-glucopyranosylapigenin (isoschaftoside), in the root extract and root exudates of *D. uncinatum* that affect the early stages of *Striga* development.[6]

Elucidating the biosynthetic pathway for this class of compound and characterising the enzymes that control *C*-glycosylflavonoid biosynthesis provides the potential for transferring the mechanism for *Striga* protection from this cattle forage legume into an edible crop legume,[6] and *C*-glycosyltransferase (CGT) activity is a key role. Previous biosynthetic studies have shown incorporation of radiolabelled naringenin and *p*-coumaric acid but not apigenin into *C*-glucosylflavones by *Swertia japonica* (Gentianaceae)[7] and *Spirodela polyrhiza* (Lemnaceae).[8,9] This work used whole plants and did not preclude the possibility of a 2-hydroxyflavanone as the glucosylation substrate. Later work showed incorporation of 2-hydroxyflavanones into *C*-glucosylflavones by a protein preparation from buckwheat cotyledons, *Fagopyrum esculentum* (Polygonaceae)[10,11] and more recently in wheat, *Triticum aestivum* L. (Poaceae) and rice, *Oryza sativa* (Poaceae).[12]

To study *C*-glycosylflavonoid biosynthesis in *D. uncinatum* we completed a total synthesis of 2-hydroxynaringenin (**1**) using a rearrangement as the key to creating the carbon skeleton (Scheme 1). 6-Hydroxy-2,4-dibenzyloxyacetophenone (**2**) was prepared by benzylation of triacetoxyphloroglucinol[13] and acylation followed by monodeprotection. Esterification with 4-benzyloxybenzoic acid gave **3**, a substrate that yields **4** after the Baker–Venkataraman rearrangement. Deprotection produces 2-hydroxynaringenin (**1**) which could be further converted into apigenin (**5**) by dehydration in mild acid and this was hydrogenated to naringenin (**6**), with a side product being dihydronaringenin chalcone. NMR analysis of **1** was performed at −60 °C in acetone at which temperature the interconverting ring-closed and open-chain tautomers were distinct on the NMR timescale.[14] The reaction sequence was repeated using [2,3,5,6-$^2H_4$]-4-benzyloxybenzoic acid prepared from [2,3,4,5,6-$^2H_5$]-phenol (Scheme 2)[15] to yield the labelled materials [2′,3′,5′,6′-$^2H_4$]-**1**, [2′,3′,5′,6′-$^2H_4$]-**5** and [2′,3′,5′,6′-$^2H_4$]-**6**.

Incubation of [2′,3′,5′,6′-$^2H_4$]-**1** with UDP-glucose and a protein extract isolated from *D. uncinatum* leaves was performed.[16,17] The organic residue was purified by HPLC to afford vitexin (8-*C*-β-D-glucopyranosylapigenin) and isovitexin (6-*C*-β-D-glucopyranosylapigenin) which were produced during the assay. Electrospray mass spectrometry showed that during the incubation with leaf protein [2′,3′,5′,6′-$^2H_4$]-**1** was converted into

* Corresponding author. Tel.: +44 1582 763133; fax: +44 1582 762595.
E-mail address: tony.hooper@bbsrc.ac.uk (A.M. Hooper).

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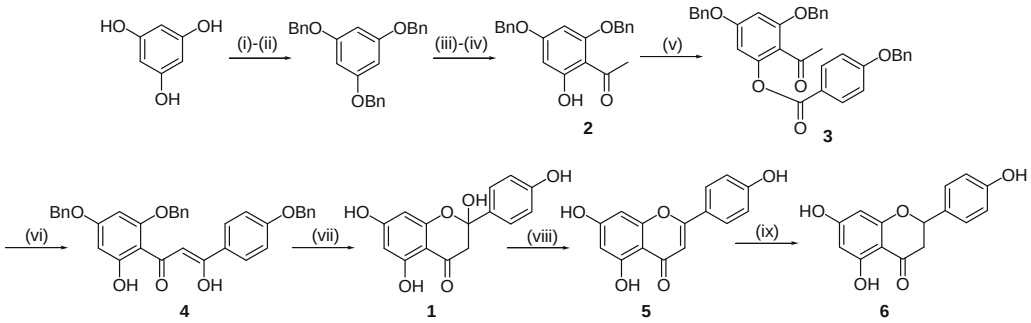
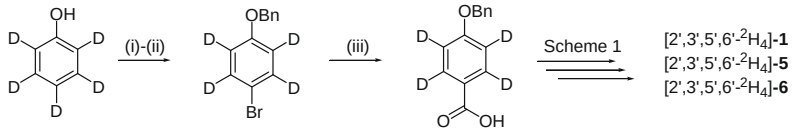


Scheme 1. Synthesis of 2-hydroxynaringenin (**1**). Reagents and conditions: (i) Ac_2O, pyridine; (ii) NaH, BnCl, H_2O, quant. 2 steps; (iii) AcOH, TFAA, 0 °C, 87%; (iv) $TiCl_4$, 76%; (v) water-soluble DCC, 4-benzyloxybenzoic acid, DMAP; (vi) KOH, pyridine, 75 °C, 67%; (vii) H_2, 10% Pd/C, 65%; (viii) MeOH, few drops 3 N HCl, quant.; (ix) H_2, 10% Pd/C, 25%.



Scheme 2. Synthesis of [2,3,5,6-$^2H_4$]-4-benzyloxybenzoic acid. Reagents and conditions: (i) Br_2, dioxane; (ii) K_2CO_3, BnBr, 63% over 2 steps; (iii) Mg, CO_2, 41%.

both [2′,3′,5′,6′-$^2H_4$]-vitexin and the regioisomer [2′,3′,5′,6′-$^2H_4$]-isovitexin (Fig. 1). A small quantity of unlabelled vitexin and isovitexin was present in the labelling experiments and protein controls as they are water soluble and co-extracted with the leaf proteins. In further experiments [2′,3′,5′,6′-$^2H_4$]**-5** and [2′,3′,5′,6′-$^2H_4$]**-6** were not converted into [2′,3′,5′,6′-$^2H_4$]-vitexin (Fig. 2) and so are not C-glucosylation substrates. There was no conversion of [2′,3′,5′,6′-$^2H_4$]**-1** into vitexin or isovitexin in the controls without the addition of UDP-glucose, nor in experiments containing protein denatured by boiling water, thereby demonstrating glucosylation to be enzyme-mediated.

[2′,3′,5′,6′-$^2H_4$]**-1** exists as a mixture of tautomers and either the open-chain or the ring-closed form may be C-glucosylated. The nucleophilicity of the aromatic ring to facilitate C-glucosylation could be increased by a phenolate ion stabilised by the keto-enol form of the open-chain [Fig. 3 (inset)], by analogy to the proposed mechanism of *Streptomyces fradiae* *C*-glycosyltransferase, UrdGT2, in which the Asp137 residue removes a phenolic proton, stabilising the substrate and increasing the nucleophilicity.[18] [2′,3′,5′,6′-$^2H_4$]**-1** is a symmetric substrate when in the open-chain form and also after glucosylation, so the production of both *C*-glucosylflavones, vitexin and isovitexin, may result either from chemical dehydration of the symmetric glucosylated intermediate or by dehydration controlled by the *C*-glycosyltransferase (CGT) or a CGT-independent dehydratase.

The substrate for C-glucosylation has been demonstrated to be **1** in Poacaea[12] and Polygonaceae,[10,11] and other work in the Lemnaceae[8,9] and Gentianaceae[7] is consistent. We have completed a high yielding total synthesis of **1**, which can be adapted to produce substrate analogues, and used stable isotopic labelling of **1** to demonstrate that it is the C-glucosylation substrate for the biosynthesis of *C*-glucosylflavones vitexin and isovitexin in *D. uncinatum* (Faba-

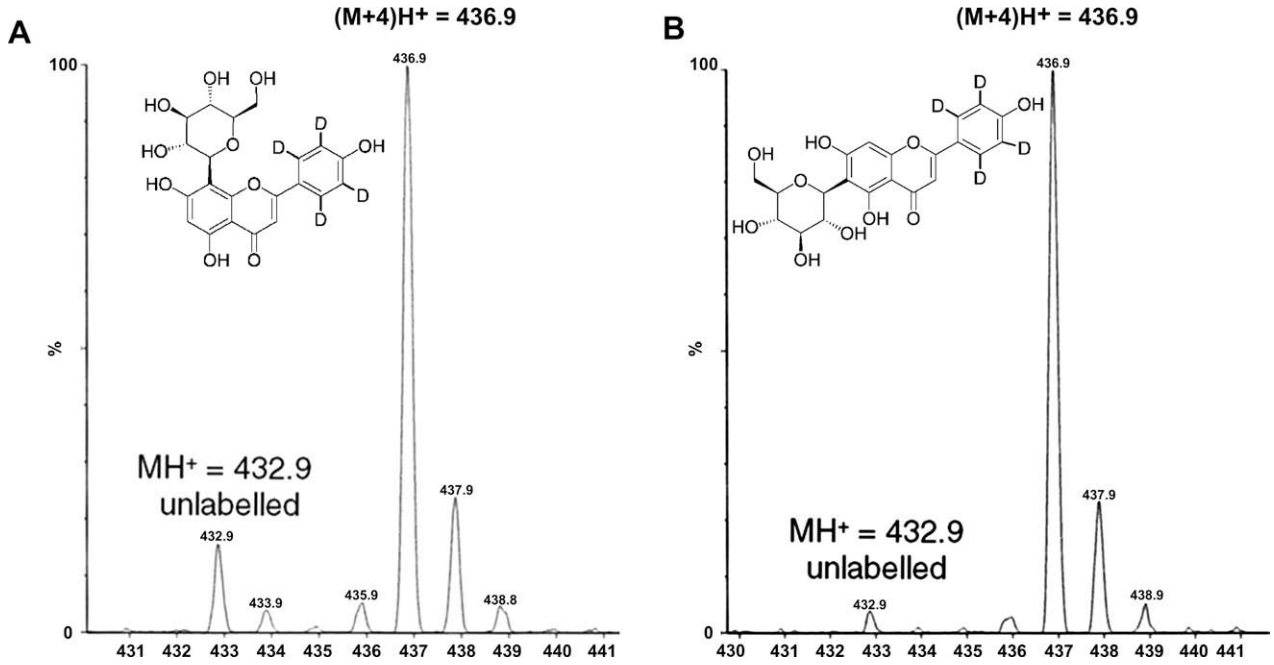


Figure 1. ESMS spectra of HPLC-purified vitexin (**A**) and isovitexin (**B**) from crude *D. uncinatum* leaf protein incubated with UDP-glucose and [2′,3′,5′,6′-$^2H_4$]**-1**.

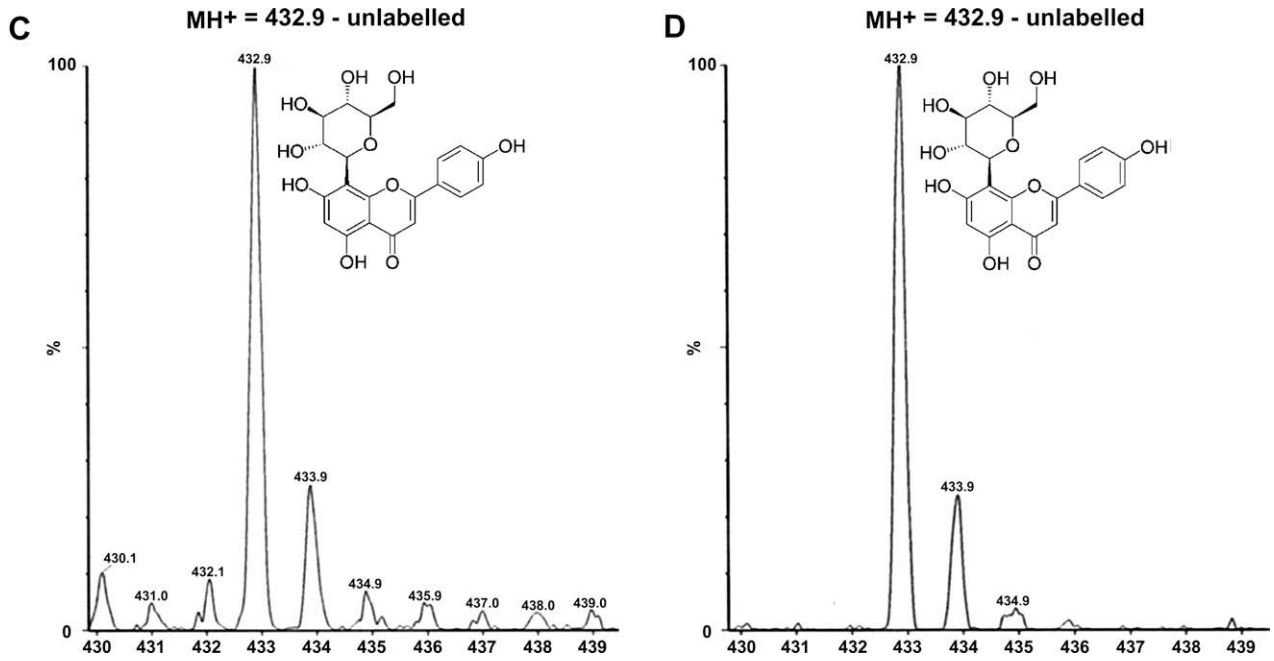


Figure 2. ESMS spectra of HPLC-purified vitexin from crude *D. uncinatum* leaf protein incubated with UDP-glucose and (**C**) [2′,3′,5′,6′-$^2H_4$]**-5** or (**D**) [2′,3′,5′,6′-$^2H_4$]**-6**.

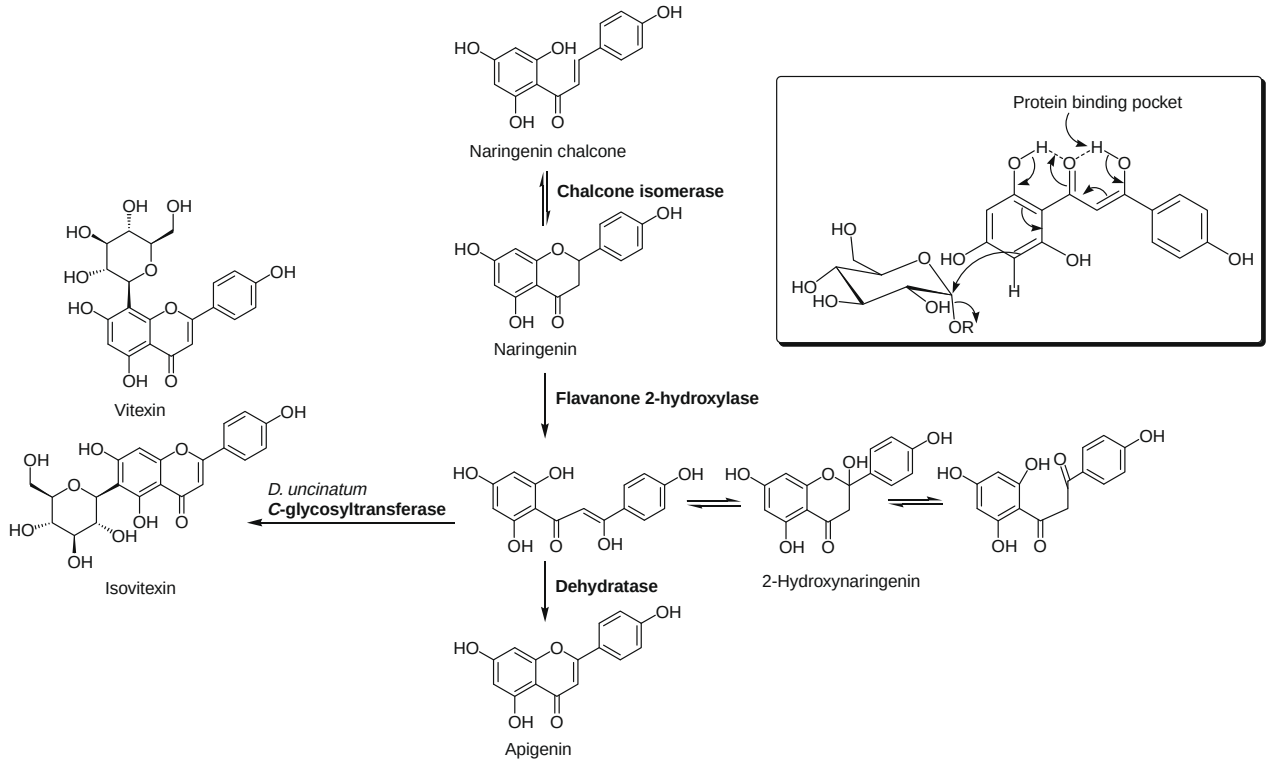


Figure 3. Biosynthetic pathway for *C*-glucosylflavonoid biosynthesis in *D. uncinatum* and (inset) putative mechanism *via* the keto-enol tautomer of 2-hydroxynaringenin (**1**).

ceae). This further supports a general biosynthetic pathway for *C*-glucyosylflavones in plants (Fig. 3).

Acknowledgements

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16. Materials and methods: *Protein isolation*: *D. uncinatum* seeds were obtained from Western Seed Company, Kitale, Kenya. Plants were grown in a glasshouse with an 8-h dark/ 16-h light cycle with day and night temperatures of 20 and 18 °C in Rothamsted Standard Compost Mix. Crude protein extract was isolated according to Brazier and Cole[17] with some modifications. Leaves were harvested, weighed, snap frozen in liquid N_2 and ground to a powder with a pestle and mortar. The powder was extracted on ice with 5 v/w of 0.1 M HEPES–NaOH, pH 7.2, containing 2 mM dithiothreitol (DTT) and 0.025 g/mL of polyvinylpolypyrrolidone (PVP). The mixture was filtered through muslin prior to centrifugation at 8000*g* for 30 min. Protein was precipitated between 45–65% $(NH_4)_2SO_4$ saturation and the pellet recovered by centrifugation at 27000*g* for 60 min. Protein pellets were frozen in liquid N_2 and stored at −80 °C.
Incubation: Enzyme analysis was performed according to Brazierand Cole[17] with modifications. Protein pellets were desalted in 0.2 M HEPES–NaOH, pH 8, containing 2 mM DTT using Sephadex G-25 gel filtration chromatography (PD-10 columns, GE Healthcare). Protein content was determined using Bradford reagent according to the manufacturer's instructions (Sigma). Protein extract was diluted to 2 mg/mL in buffer and pre-incubated at 30 °C with 66 μM [2′,3′,5′,6′-$^2H_4$]-**1**, -**5** or -**6** for 3 min. The reaction was initiated by the addition of 100 μM UDP-glucose, stopped after 45 min by the addition of 0.3 M HCl (250 μl) and extracted with EtOAc, 1.5 mL/mg protein. The organic layer was evaporated under N_2 and re-suspended in MeOH.
HPLC: Samples were isolated on an ACE 5 AQ column (250 mm × 4.6 mm), using a mobile phase comprising two solvents, solvent A (H_2O–HCO_2H 95:5) and solvent B (MeOH). Samples were eluted with a series of linear gradients set initially at 95:5 A:B (0 min), 85:15 (3 min), 75:25 (13 min), 70:30 (25 min), 45:55 (35 min), 45:55 (45 min), 5:95 (46 min), 5:95 (55 min) and 95:5 (60 min).
ESMS: Purified fractions were collected from the HPLC column, evaporated under N_2 and re-suspended in MeOH prior to analysis by ESMS using an Autospec Ultima Mass Spectrometer [Waters Corp (Micromass UK)] equipped with an electrospray ionisation source (ESI). Positive ion mode ESI-MS was performed at 4 kV and 80 °C, with N_2 gas as the desolvation and nebuliser gas (350 L/h and 15/20 L/h). The solvent used as mobile phase for ESI-MS was MeOH–H_2O (1:1) with 2% AcOH.
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