Phytochemistry, 1978, Vol 17, pp. 1683–1684. © Pergamon Press Ltd Printed in England. 0031-9422/78/0901-1683 $02 00/0

IDENTIFICATION OF ISOFLAVONES CALYCOSIN AND PSEUDOBAPTIGENIN IN *TRIFOLIUM PRATENSE*

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(*Received* 15 *February* 1978)

Key Word Index—*Trifolium pratense*; Leguminosae; red clover; isoflavones; calycosin; pseudobaptigenin.

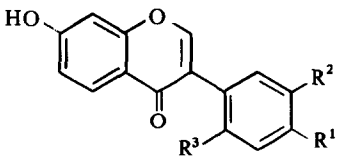
This paper reports the detection and identification of two 3',4'-dioxygenated isoflavones from red clover: 3'7-dihydroxy-4'-methoxyisoflavone (calycosin) (**1**) and 7-hydroxy-3',4'-methylenedioxyisoflavone (pseudobaptigenin) (**2**). Both isoflavones have previously been reported from a variety of other sources [1–3], but neither has yet been detected in pasture legumes. Their detection in red clover has important implications in the biosynthesis of the pterocarpan phytoalexin (6aR, 11aR)-maackiain (**10**) and the corresponding coumestan, medicagol (**12**) in this plant.

Pseudobaptigenin (**2**) was detected as a minor component (*ca* 5%) in a bulk crystalline preparation of formononetin (**3**). No separation of (**2**) and (**3**) was achieved by TLC or PC, using a variety of solvents, including those reported previously to separate methoxy- and methylenedioxy-substituted pterocarpans [4]. The mixture was resolved only by GLC, and the identity of the minor component confirmed as (**2**) by GC–MS of the TMSi derivatives, by comparison with an authentic sample.

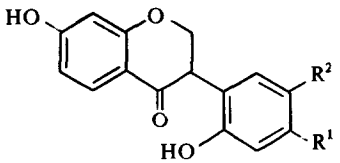
Calycosin (**1**) was isolated from the mother liquor and crystallised. PMR in DMSO-d6 indicated a pattern characteristic of 7,3',4'-hydroxylation [5] and in agreement with that described previously [1]. Isoflavone (**1**) was also distinguished from a previously reported isomeric form (4',7-dihydroxy-3'-methoxy-isoflavone (**4**)) [6] by a positive Gibbs test [7]. Isoflavones (**1**) and (**2**) were also detected in ether extracts of freeze-dried plant material, which had not been exposed to acid or alkali during the extraction procedure. It was therefore considered unlikely that (**1**) and (**2**) are artefacts of plant preparation or of isolation.

The detection in red clover of isoflavones (**1**) and (**2**) with *ortho* oxygenation in ring B is of considerable biosynthetic interest in the light of feeding experiments recently reported by Dewick [8]. In Cu^{2+}-treated red clover seedlings, formononetin (**3**) was incorporated into both pterocarpans maackiain (**10**) and medicarpin (**11**). However, 2'-hydroxyformononetin (**6**) and its corresponding isoflavanone (**8**) were incorporated into (**11**) only and not into (**10**) [8]. Nor was (**11**) itself incorporated to any significant extent into (**10**) [8]. These results imply that formation of the methylenedioxy moiety of (**10**) must precede 2'-hydroxylation.

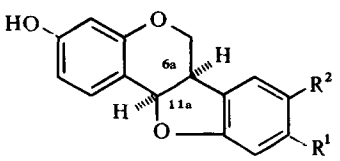
Our results show that red clover does in fact contain a methylenedioxyisoflavonoid lacking a 2'-hydroxyl group, namely (**2**). Further, (**1**) has already been suggested to be a likely precursor of (**2**) [1]. That the co-occurrence of (**1**) and (**2**) with (**10**) in red clover may be more than fortuitous is also suggested by earlier reports of the co-occurrence of (**1**) and (**10**) in *Pterocarpus* spp. [2]



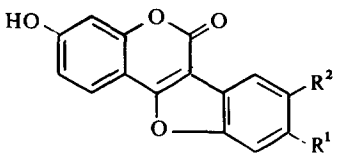
	R^1	R^2	R^3
(**1**)	OMe	OH	H
(**2**)	O—CH_2—O		H
(**3**)	OMe	H	H
(**4**)	OH	OMe	H
(**5**)	OH	H	H
(**6**)	OMe	H	OH
(**7**)	O—CH_2—O		OH



(**8**) R^1 = OMe, R^2 = H
(**9**) R^1, R^2 = O—CH_2—O



(**10**) R^1,R^2 = O—CH_2—O
(**11**) R^1 = OMe, R^2 = H



(**12**) R^1, R^2 = O—CH_2—O

and of (2), (10) and sophorol (9) in *Maackia* spp. [9]. Dewick has suggested that his data are consistent with the biosynthetic relationship: (3) → (6) → (8) →→ (11) [8]. Although the possibility of a metabolic grid cannot be excluded in either pathway, the detection of (1) and (2) in red clover means that an analogous scheme can now be written for the pathway of biosynthesis of (10) in this plant: (3) → (1) → (2) → (7) →→ (9) →→ (10). Initial steps in the biosynthesis of (12) are likely to be similar.

EXPERIMENTAL

All mps are uncorrected. MS were recorded at 70 eV. GC–MS of TMSi derivatives (BSA, pyridine [10]) was carried out on 3% OV–101 using a glass column (6 m × 2 mm) at He flow rate 60 ml/min; injection temp. 260°; column 230°; FID 260°; membrane 200°. Retention times are quoted relative to formononetin (9.5 min).

Extraction procedure

Red clover leaves and stems (1.3 kg dry wt, cv Grasslands Hamua) were dried at 37° and extracted with MeOH. Subsequent handling was essentially as described previously [11] except that glycosides were acid hydrolysed (H_2SO_4, 48 hr room temp.) prior to solvent fractionation (EtOAc), and phenolic materials were extracted first from EtOAc with 1 M NaOH and then into Et_2O after re-acidification. The resultant extract was chromatographed on Al_2O_3 (750 g, Woelm, neutral, activity grade 1), with $CHCl_3$, EtOH and EtOH:H_2O (1:1). Fractions rich in formononetin (TLC) were combined, crystallised, yielding needles (5.27 g): mp (EtOH) 259–260°; MS (probe) *m/e* (rel. int.) 282 (5), 268 (100), λ_{max}^{MeOH} nm (log ε): 249 (4.44), 300 (4.02); cf. (3) lit. [12, 13].

Formononetin (3) *and pseudobaptigenin* (2). GC–MS analysis of the TMSi derivative of the crystalline product showed the presence of (3) (95%); *m/e* (rel. int.) 340 (M^+; 100), 325 (35), 208 (14), 132 (41); and (2) (5%); RR_t 1.37; *m/e* (rel. int.): 354 (M^+; 100), 339 (22), 146 (51), which co-chromatographed with and gave identical MS characteristics to the TMSi derivative of authentic pseudobaptigenin derived from pseudobaptisin by β-glucosidase hydrolysis. Authentic pseudobaptigenin: mp 291–293° (lit [12], 292°); MS (probe) *m/e* (rel. int.) 282 (M^+; 100), 146 (95).

Calycosin (1) was separated from the mother liquor of crystallisation (0.32 g) by PC with C_6H_6–HOAc–H_2O (125:72:3). Material R_f 0.4–0.5 (colour reaction as in [1]) was eluted and crystallised (29 mg) mp (EtOH–H_2O) 249–251° (lit. [2] 245–247°) MS (probe) *m/e* (rel. int.) 284 (M^+; 100), 269 (26), 241 (23), 213 (26), 148 (12), 137 (39); λ_{max}^{MeOH} nm: 248, 258 (*sh*), 291, 313 (*sh*); $\lambda_{max}^{MeOH+NaOH}$ nm: 257, 332; PMR (60 MHz, DMSO-d6, TMS): δ 8.30 (1 H, *s*), 8.01 (1 H, *d*, *J* = 9 Hz), 6.87–7.17 (5 H, *m*), 3.80 (3H, *s*) lit. [1]. GC–MS (TMSi derivative): RR_t 1.82; *m/e* (rel. int.) 428 (M^+; 100), 413 (15).

Other components of the mother liquor were identified and quantities estimated by PC and GC–MS of TMSi derivatives as (3) 51%; daidzein (5) (RR_t 1.37) 37%; (1) 12%; (2) <0.5% and a trace of coumestan tentatively identified by MS as medicagol (12) (RR_t 2.37).

Acknowledgements—The authors are grateful to Dr K. Markham of the Chemistry Division, DSIR, for a gift of pseudobaptisin, to Mr P. Ellingham for MS and GC–MS, to Miss P. Dittmer for technical assistance and to Dr J. Lancashire of the Grasslands Division, DSIR for samples of field-grown red clover.

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Phytochemistry, 1978, Vol 17, pp 1684–1685 © Pergamon Press Ltd Printed in England 0031-9422/78/0901 1684 $02.00/0

EIN BUTYRYL-FLAVONOL AUS DEM MEHL VON *NOTHOLAENA AFFINIS*

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(*Eingegangen am* 1. *März* 1978)

Key Word Index—*Notholaena affinis*; Polypodiaceae; farinose exudate; novel flavonol with butyryl side-chain; 7,4′-*O*-dimethyl-8-*O*-butyrylherbacetin.

Notholaena affinis (Mett.) Moore gehört zu denjenigen Vertretern dieser Gattung, die Flavonoid-Aglyka in mehlartiger Form extern ablagern. Als Verbreitungsgebiet wird Mexiko und Mittelamerika angegeben [1]. Im Rahmen einer umfassenden Untersuchung mehlbildender Farne [vgl. 2, 3] fiel eine Population von *N. affinis* aus Costa Rica durch die Anwesenheit mehrerer unbekannter Substanzen auf. Die Hauptkomponente des Exkrets konnten wir jetzt identifizieren als 7,4′-*O*-dimethyl, 8-*O*-butyryl Derivat des Herbacetins.

Das hellgelbe Mehl auf den Farnwedeln läßt sich mit Aceton und Benzol ablösen und liefert die als *NA-1*