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AN 8-HYDROXYLATED EXTERNAL FLAVONE AND ITS 8-*O*-GLUCOSIDE FROM *BECIUM GRANDIFLORUM*

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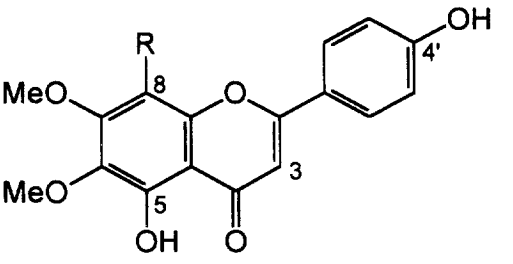
Key Word Index—*Becium grandiflorum*; Lamiaceae; flavonoid glycosides; external flavonoids; isothymusin 8-*O*-glucoside; isothymusin; cirsimaritin; chemotaxonomy.

Abstract—The major vacuolar flavonoid of a greenhouse-grown plant of *Becium grandiflorum* has been identified as the 8-*O*-glucoside of isothymusin (5,8,4′-trihydroxy-6,7-dimethoxyflavone), while the major external flavonoids in the diethyl ether surface wash of the same plant are found to be isothymusin and cirsimaritin (5,4′-dihydroxy-7,8-dimethoxyflavone). Although isothymusin is already known as a conversion product of thymusin (5,6,4′-trihydroxy-6,7-dimethoxyflavone) formed by means of the Wessely–Moser rearrangement, this is the first report of isothymusin as a plant constituent, both as aglycone and glycoside. The biogenetic and chemotaxonomic implications of the occurrence of these flavonoids in *B. grandiflorum* are briefly discussed.

INTRODUCTION

The genus *Becium* Lindl. (Lamiaceae) comprises *ca* 33 species and is distributed in tropical Africa, Arabia and India [1]. It is closely related to *Ocimum* (basil) with no sharp distinction evident between the two genera. *B. grandiflorum* (Lam.) Pic. Serm. is a perennial aromatic shrub up to 2.5 m tall, with pubescent or tomentose stems and leaves, and occurs in Kenya, Ethiopia and Tanzania. To the best of our knowledge no reports have yet been published on the chemistry of this plant. As part of a chemotaxonomic survey of *Ocimum* and related genera we report here on the identification of the major vacuolar and surface flavonoids from *B. grandiflorum*.



1 R = OGlc
2 R = OH
3 R = H

RESULTS AND DISCUSSION

The major flavonoid glycoside **1** from the methanol extract of *B. grandiflorum* leaves (extract 3, see Experimental) was isolated and purified by means of preparative PC and semi-preparative HPLC. Its molecular structure was determined principally by ^{1}H NMR techniques, including the use of excitation sculpted singly selective 1D TOCSY (XSTOCSY) and ROESY (XSROESY) experiments [2]. These are of particular value when the amount of sample is limited, as in the case of **1**, where no more than 1 mg could be obtained from the small quantity of plant material available for extraction. The ^{1}H NMR spectrum contained several distinctive resonances, including an exchangeable 1H singlet at δ 13.02, indicative of a free 5-hydroxyl group, two 2H doublets at δ 8.09 (J = 8.8 Hz) and 6.83 (J = 8.8 Hz), a 1H singlet at δ 6.79, and two 3H singlets at δ 4.02 and 3.83 typical of methoxyl groups. The aromatic resonances are characteristic of a B ring substituted at the 4′ position and a single isolated proton at either C-3 or in the A ring. An additional 1H doublet at δ 4.85 (J = 7.6 Hz) and a subset of resonances between 3.10 and 3.65 ppm suggested the presence of a glycosyl group. All the glycosyl resonances in the ^{1}H spectrum were assigned from COSY and HSQC data, which also provided the cor-

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responding ^{13}C assignments. The sequential connectivities, chemical shift parameters and coupling constant data confirmed that the glycosyl unit was an *O*-linked glucose with the β-configuration [3, 4]. Selective excitation of the anomeric H-1″ proton in a 1D XSTOCSY experiment showed that TOCSY transfer could be achieved as far as the C-6″H_2 protons. The correct placing of the substituents on the flavonoid skeleton, and thereby the identity of the compound, was obtained from two singly selective 1D XSROESY experiments. Selective excitation of the methoxyl proton resonance at δ 3.83 gave only a single ROE to the methoxyl protons at δ 4.02. This indicated that the methoxyl group corresponding to the δ 3.83 resonance could not be in close proximity to any protons other than those of the second methoxyl group. The two methoxyl groups must, therefore, be adjacent substituents on the A ring In a second experiment, selective excitation of the anomeric H-1″ resonance at δ 4.85 gave ROEs to the B ring aromatic protons at δ 8.09, the methoxyl protons at δ 4.02, and a number of glucosyl proton resonances. The glucosyl substituent must, therefore, be adjacent to one methoxy group, but more importantly, located sufficiently close to the B ring for a ROE to be observed with the H-2′,6′ protons which correspond to the δ 8.09 resonance. These conditions can only be satisfied by placing the glucosyl substituent at C-8. The two methoxyl groups at δ 4.02 and 3.83 must therefore be situated at C-7 and C-6, respectively, while the C-5 position is already known to be substituted by a hydroxyl group. The aromatic resonance at δ 6.79 can be assigned to H-3, as expected. Compound **1** is therefore 8-*O*-β-D-glucopyranosyloxy-5,4′-dihydroxy-6,7-dimethoxyflavone, a new flavone glycoside.

The UV spectrum of **1** in MeOH closely resembled that of a standard of 5,4′-dihydroxy-6,7,8-trimethoxyflavone (xanthomicrol) with its characteristically wide band II. As expected, the use of shift reagents indicated that the 7-hydroxyl was blocked, the 5-hydroxyl was free and that no vicinal dihydroxyl groups were present in either the A or B rings [5]. Acid hydrolysis of **1** gave glucose and a mixture of one major and one minor aglycone. The presence of two aglycones is a consequence of the acid-catalysed interconversion of 6- and 8-hydroxylated flavones often referred to as the Wessely–Moser rearrangement. Thus, it is to be expected that hydrolysis of **1** will give a mixture of 5,8,4′-trihydroxy-6,7-dimethoxyflavone and 5,6,4′-trihydroxy-7,8-dimethoxyflavone. The latter compound has been isolated previously from *Thymus membranaceus* Boiss. and given the common name thymusin [6]. Its 8-hydroxy isomer, isothymusin, is known only as an acid-catalysed interconversion product of thymusin [6]. Therefore, compound **1** may be referred to as isothymusin 8-*O*-glucoside. Treatment of a standard of thymusin under acidic conditions followed by HPLC analysis of the reaction products indicated that two aglycones were present as anticipated. One of these gave a sharp well-defined peak and a UV spectrum corresponding to that of isothymusin [6]. This has a characteristic band III with a λ_{max} of 306 nm indicative of a free 8-hydroxyl group in an apigenin derivative [7]. The second aglycone gave a broad rather undefined peak with a UV spectrum identical to that of thymusin [6]. A summary of the data concerning retention times and UV spectral parameters is given in Table 1. The HPLC trace of the ethyl acetate fraction of acid-hydrolysed **1** also exhibited two peaks with the same R_fs and peak shapes as those of acid-treated thymusin. The compound corresponding to the major peak had the same UV spectrum as that of isothymusin as expected, but no UV spectrum could be obtained for the minor component due to its low concentration. It is useful to note that the UV spectrum of **1** does not show the characteristic band III at 306 nm as in isothymusin, because the 8-hydroxyl group is blocked.

HPLC traces of the diethyl ether extracts 1 and 2 (see Experimental) were dominated by two peaks representing compounds **2** and **3**. These exhibited UV spectra typical of flavones. The concentration of the compounds was highest in extract 1 (diethyl ether rinse for a few seconds), indicating that both **2** and **3** were surface flavonoids. Compound **3** was identified directly in the crude extract by means of co-chromatography (HPLC) with an authentic standard as cirsimaritin (5,4′-dihydroxy-6,7-dimethoxyflavone). R_fs and spectral characteristics in the two solvent systems used are given in Table 1. Compound **2**, which was present in the extract at approximately twice the concentration of **3**, had the same retention time and characteristic UV spectrum as isothymusin derived from acid-treated thymusin (Table 1). Attempts to purify **2** by means of preparative TLC or semipreparative HPLC met with little success due to the rapid decomposition of the compound during these isolation procedures. However, acidic treatment of **2** yielded the same two compounds as acidic treatment of thymusin and acid hydrolysis of **1** (see Table 1). Therefore, it may be concluded that **2** is isothymusin.

The presence of isothymusin as an external flavonoid in a species belonging to the family Lamiaceae and in a genus related to *Ocimum* is interesting both biogenetically and chemotaxonomically. Although 8-methoxylated surface flavones are quite common in this family, and are likely to have the corresponding 8-hydroxylated derivatives as precursors, accumulation of 8-hydroxylated external flavones is rare and has only been reported from the genus *Scutellaria*. However, in that genus the compounds have either an unsubstituted B ring or a hydroxyl group at the 2′-position. They do not have a hydroxyl group at the 4′-position as in isothymusin [8]. The presence of isothymusin in *B. grandiflorum* is not unreasonable, since this compound could be formed by hydroxylation at C-8 of cirsimaritin (**3**), the second most abundant external flavone in the plant. Cirsimaritin is also common in the related genus *Ocimum* [9]. Isothymusin is presumably a precursor of 5,4′-dihydroxy-6,7,8-tri-

Table 1. HPLC R_ts and UV spectra (photodiode array detector) of *Becium grandiflorum* flavonoids and standards before and after treatment under acidic conditions

Compound	R_t in solvent I (min)	R_t in solvent II (min)	λ in nm
1	13.7	17.5	281, 300sh, 338
1 After acid hydrolysis	17.6#	n.d.	285sh, 306, 334, 360i
	19.0+	n.d.	—*
2	17.6	20.5	285sh, 306, 334, 360i
2 After acid treatment	17.6#	n.d.	285sh, 306, 334, 360i
	19.0+	n.d.	—*
Thymusin	19.0+	n.d.	297, 337
Thymusin after acid treatment	17.6#	n.d.	285sh, 306, 334, 360i
	19.0+	n.d.	297, 337
3	19.4	21.9	275, 336
Cirsimaritin	19.4	21.9	275, 336

sh = shoulder; i = inflexion point; n.d. = not determined; # sharp, well defined peak; + broad, poorly defined peak; * amounts too small to give a UV spectrum. The composition of solvent systems I and II is given in the Experimental Section.

methoxyflavone (xanthomicrol), which we have found in *O. americanum* var. *americanum* (R. J. Grayer *et al.*, unpublished results), but not in *O. basilicum* [9], despite an earlier claim to the contrary [10]. In *Ocimum* species it is expected that an 8-*O*-methyltransferase catalyses the conversion of isothymusin to xanthomicrol, whereas this enzyme is absent or inactive in *B. grandiflorum*. On the other hand, *B. grandiflorum* should possess a 8-*O*-glucosyltransferase in order to produce the 8-*O*-glucoside of isothymusin. It is noteworthy that a methoxylated flavone such as isothymusin occurs both as a surface flavonoid and a glycoside in the same plant, since such co-occurrence is rare in Lamiaceae. Exceptions include cirsimaritin, salvigenin and cirsiliol, which are known to co-occur with glycosidic forms in *Rosmarinus officinalis*, *Salvia* species and *Teucrium arduini*, respectively [11]. The 3′-hydroxylated derivative of **1**, leucanthogenin 8-*O*-glucoside, has been reported from another labiate, *Sideritis leucantha* [12], but in this case the free aglycone was not found. It is likely that **1** belongs to that class of monoglycosylated methoxylated flavonoids which are intermediate in polarity between lipophilic surface flavonoids and polar vacuolar flavonoids, and are thought to be located in the walls of epidermal cells [13]. They may have a similar function to external flavonoids.

EXPERIMENTAL

Plant material. Plants of *B. grandiflorum* were grown under greenhouse conditions at the Royal Botanic Gardens (RBG), Kew (accession number 1992-1962). These were raised from seeds collected in Ethiopia (Tigre, Mekele to Abergele) by Dr A. Paton, RBG, Kew, who also verified the plant material. A voucher specimen of the plant material has been deposited in the Herbarium, RBG, Kew.

General. ^{1}H NMR spectra were recorded at 500 MHz. Samples were dissolved in DMSO-d_6 with TMS as primary reference. A temp. of 37° was used for all NMR experiments.

Extraction procedures. 17 g of freeze-dried leaves (stalks removed) from greenhouse-grown plants was immersed in 600 ml Et_2O, and the solvent immediately poured off, in order that only external compounds should be dissolved. The solvent was filtered and evapd to dryness by means of rotary evapn, and the residue dissolved in a known volume of 80% MeOH (extract 1). The leaves were subsequently extracted in a second batch of 600 ml Et_2O for 24 hr. This extract was also filtered, evapd to dryness and dissolved in 80% MeOH (extract 2). The leaves were extracted for a third time, but now with 100% MeOH. After 24 hr, this extract was evapd to dryness and the residue dissolved in a known amount of 80% MeOH (extract 3). All three extracts were analysed by means of HPLC with photodiode array detection and used for subsequent isolation of flavonoids.

Isolation of flavonoids. The flavone glycoside **1** was isolated by two prep. PC steps using *n*-BuOH–H_2O–HOAc (4:1:5, upper layer) and 15% aq. HOAc as solvents, respectively, followed by semiprep. HPLC of the purified glycoside fr.

Analytical and semi-prep. HPLC. A Waters HPLC system consisting of a LC 600 pump and 996 photodiode array detector was used in gradient elution mode. For analytical HPLC, a LiChrospher 100RP-18 column, 4.0 mm (i.d.) × 250 mm was used with 2% HOAc (A) and MeOH–HOAc–H_2O (18:1:1) (B) as eluting solvents. Initial conditions for solvent systems I and II were A = 60% and A = 75% respectively, with a linear gradient reaching A = 0% at t = 20 min.

Isocratic elution continued to $t = 25$ min, after which time the programmes returned to the initial solvent compositions. A flow rate of 1.0 ml min^{-1} and a column temp. of 25° were maintained throughout. R_fs of flavonoids recorded with sample injection carried out by an autosampler were *ca* 2 min longer than those recorded previously with identical soln conditions but using manual injection [9]. Semiprep. HPLC isolations were carried out using a LiChrospher 100RP-18 column, 10.0 mm (i.d.) × 250 mm. A gradient elution programme similar to system II was used, except that solvents A and B were H_2O and MeOH, respectively, thus avoiding contamination of the isolated frs with HOAc. A flow rate of 4.5 ml min^{-1} and a column temp. of 30° were maintained.

Acid hydrolysis and treatment of aglycones. Aliquots of **1**, **2** and the standard of thymusin were dissolved in 0.5 ml 80% MeOH in a test tube, and 3 ml of 2 M HCl added. This mixt. was heated in a boiling H_2O bath for 1 hr. The resulting soln was cooled and extracted with 2 ml of EtOAc. The dried EtOAc extract was dissolved in 0.8 ml of 80% MeOH and analysed by HPLC for flavone aglycones. The aqueous layer from **1** was also evapd to dryness and analysed by PC for sugars [14].

Isothymusin 8-*O*-*glucoside* (**1**). UV λ_{max} MeOH nm: 275, 294sh, 332; +NaOH 271, 312sh, 356, 392sh; +$AlCl_3$ 278, 306, 348; +$AlCl_3$ and HCl 278, 306, 348; +NaOAc 271, 334sh, 394; +NaOAc and H_3BO_3 275, 294sh, 334. (Note that the UV spectrum generated by the photodiode array detector (Table 1) differs slightly from the MeOH spectrum due to the presence of HOAc in the eluting solvent). ^1H NMR (DMSO-d_6): δ 13.02 (1H, *s*, 5-OH), 8.09 (2H, *d*, $J = 8.8$ Hz, H-2′,6′), 6.83 (2H, *d*, $J = 8.8$ Hz, H-3′,5′), 6.79 (1H, *s*, H-3), 4.85 (1H, *d*, $J = 7.6$ Hz, H-1″), 4.02 (3H, *s*, 7-OMe), 3.83 (3H, *s*, 6-OMe), 3.62 (1H, *dd*, $J = 11.6$, 1.2 Hz, H-6″), 3.39 (1H, *m*, H-6″), 3.38 (1H, *m*, H-2″), 3.26 (1H, *m*, H-3″), 3.18 (1H, *m*, H-4″), 3.11 (1H, *m*, H-5″). ^{13}C NMR (DMSO-d_6) (assignments of non-quaternary C atoms from HSQC): δ129.0 (C-2′,6′), 116.3 (C-3′,5′), 103.6 (C-1″), 101.3 (C-3), 76.9 (C-5″), 76.1 (C-3″), 73.9 (C-2″), 70.0 (C-4″), 61.3 (7-OMe), 60.9 (C-6″), 60.1 (6-OMe).

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