PHYTOCHEMICAL ANALYSIS
Phytochem. Anal. **16**, 334–341 (2005)
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI**: 10.1002.pca.853

Isolation and Identification of Flavonoids, Including Flavone Rotamers, From The Herbal Drug 'Crataegi Folium Cum Flore' (Hawthorn)

S. Rayyan,[1] T. Fossen,[1] H. Solheim Nateland[2] and Ø. M. Andersen[1]*

[1]Department of Chemistry, University of Bergen, Allégt. 41, N-5007 Bergen, Norway
[2]Polyphenols Laboratories AS, Hanaveien 4-6, N-4327 Sandnes, Norway

Twelve flavonoids, including seven flavones, four flavonols and one flavanone, were isolated from methanolic extract of the herbal drug 'Crataegi folium cum flore' (hawthorn leaves and flowers) by a combination of CC (over Amberlite XAD-7 and Sephadex LH-20) and preparative HPLC. Their structures, including that of the novel flavonol 8-methoxykaempferol 3-*O*-(6″-malonyl-*β*-glucopyranoside), were elucidated by homo- and heteronuclear NMR and electrospray/MS. The ^{1}H- and ^{13}C-NMR of all compounds, including rotameric pairs of five flavone *C*-glycosides, were assigned. The presence and relative proportion of each rotamer was shown by various NMR experiments, including two-dimensional nuclear Overhauser and exchange spectroscopy, to depend on solvent, linkage position and structure of the *C*-glycosyl substituent. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: NMR; nuclear Overhauser and exchange spectroscopy; flavonoids; rotamers; 8-methoxykaempferol 3-*O*-(6″-malonyl-*β*-glucopyranoside); Crataegi folium cum flore; hawthorn; Rosaceae.

INTRODUCTION

The genus *Crataegus* (hawthorn) consists of about 280 species and numerous hybrids found in northern temperate regions, including North America, Europe and northern Asia. Hawthorn is prescribed by physicians for the treatment of various conditions, including diminished heart performance, angina pectoris and mild forms of arrhythmias (German Commission, 1994; Sticher and Meier, 1998; Al Makdessi *et al.*, 1999). Hawthorn preparations may strengthen heart contractions, prevent leakage of intracellular enzymes upon ischemic injury (Ammon and Kaul, 1994; Schuessler *et al.*, 1995; Al Makdessi *et al.*, 1999) and have shown anti-viral and anti-oxidant activities (Shahat *et al.*, 2002). Many pharmacological effects of hawthorn are considered to result from its flavonoid and procyanidin content, and some commercial preparations are even calibrated with respect to the flavonoid content.

A variety of flavonoids have been reported to occur in hawthorn extracts (Sticher and Meier 1998; Melikoglu and Mericli 2000; Zhang and Xu 2003). In the present paper, the isolation of 12 flavonoids from the dried herbal drug 'Crataegi folium cum flore' by Amberlite XAD-7 column chromatography, Sephadex LH-20 gel filtration and preparative HPLC is reported. The structures of all compounds, including the novel flavonol, **1**, were mainly elucidated by a combination of homo- and heteronuclear NMR techniques and electrospray MS. The structures of all of the flavonoids, including rotameric pairs of five flavone *C*-glycosides, are supported with ^{1}H- and ^{13}C-NMR assignments. Rotameric conformers have previously been identified for proanthocyanidins (Weinges *et al.*, 1970) and several flavone *C*-glycosides (Markham *et al.*, 1987; Jay, 1993; Lewis *et al.*, 2000; Nørbæk *et al.*, 2000; Kumazawa *et al.*, 2001) including luteolin 8-*C*-*β*-glucopyranoside (**5**) and apigenin 8-*C*-*β*-glucopyranoside (**7**). The rotamers of apigenin 6-*C*-neohesperidoside (**10**), apigenin 8-*C*-neohesperidoside (**11**) and apigenin 8-*C*-(4′-acetylneohesperidoside) (**12**) have, however, not been previously reported. It is shown, by various NMR experiments, including two-dimensional nuclear Overhauser and exchange spectroscopy, that the presence of flavone rotamers and their relative proportions may depend on solvent, aglycone linkage positions and glycosyl structure.

* Correspondence to: Ø. M. Andersen, Department of Chemistry, University of Bergen, Allégt. 41, N-5007 Bergen, Norway.
Email: oyvind.andersen@kj.uib.no
Contract/grant sponsor: Norwegian Research Council.
Contract/grant sponsor: State Education Loan Fund.

EXPERIMENTAL

Plant material. The herbal drug 'Crataegi folium cum flore' (dried hawthorn leaves and flowers) was purchased from Norsk Medisinaldepot ASA (Bergen, Norway). This drug has been specified, for example, in the German, Swiss and French pharmacopoeias (DAB 10, Ph. Helv. VII, Ph Franc. X, respectively; Sticher and Meier 1998), and a specification is in preparation for the European pharmacopoeia (Ph. Eur. 3).

Extraction and purification of the flavonoids. Plant material (200 g) was extracted three times with 20% aqueous methanol at 3°C. The extracts were filtered through glass wool, concentrated under reduced pressure, purified by partition against diethyl ether, and subjected to two Amberlite (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) XAD-7 columns (75.0 × 5.5 cm i.d.). Following application of the purified concentrated flavonoid extract, the columns were washed with distilled water (6 L each) after which the flavonoids were

Received 2 July 2004
Revised 31 October 2004
Accepted 2 November 2004

eluted with methanol (Andersen, 1988). The flavonoids were further purified on a Sephadex (Amersham Bioscience AB, Uppsala, Sweden) LH-20 column (100 × 5 cm i.d.) using a step-wise gradient of methanol:water from 40:60 (v/v) to 70:30 (v/v) at a flow-rate of 2.5 mL/min. Compounds 1 and 4 were collected in a 250 mL fraction after elution of 3.1 L of solvent.

HPLC analyses. Individual flavonoids were separated by preparative HPLC using a system comprising a Gilson (Gilson, Inc. World Headquarters, Middleton, WI, USA) model 305/306 pump, a Hewlett Packard (Hewlett-Packard Palo Alto, CA, USA) HP-1040A detector and a Hypersil-ODS (Bellefonte, PA, USA) column (25 × 2.2 cm i.d.; 5 μm). The mobile phase consisted of formic acid:water (0.5:9.0, v/v; solvent A) and methanol:formic acid:water (5.0:0.5:4.0, v/v; solvent B) with the following elution profile: linear gradient from 90:10 (A:B) to 0:100 in 45 min, isocratic elution with 0:100 for the next 13 min, followed by linear gradient from 0:100 to 90:10 in 1 min. The flow-rate was 14 mL/min, and aliquots of 300 μL were injected. Altogether 26.2 mg of 1 were isolated.

Analytical HPLC was performed with an Hypersil-ODS column (20 × 0.5 cm i.d.; 5 μm) using solvents A and B as described above. The elution profile consisted of a linear gradient from 90:10 (A:B) to 0:100 in 23 min, isocratic elution with 0:100 for the next 5 min, followed by linear gradient from 0:100 to 90:10 in 1 min. The flow-rate was 0.75 mL/min and aliquots of 15 μL were injected. UV spectra were recorded on-line during the HPLC analysis over the wavelength range 240–450 nm in steps of 2 nm.

ES-MS analyses. MS measurements on compounds **1–12** were obtained by electrospray ionisation in the positive-(ESP+) mode using a Platform LCZ (Micromass, Manchester, UK). The following ion optics were used: capillary voltage +3 and +15 kV; cone voltage +15, +20 and +50 V; and extractor voltage 10 V. The source block and desolvation temperatures were 150 and 170°C, respectively. Solutions were introduced by way of a syringe pump (Pump11; Harvard Instruments) (Harvard Apparatus, Holliston, MA, USA), and the inlet flow was set to 15 mL/min. Continuous MS were recorded over the range *m/z* 250–800 with a scan time of 5 s and an interscan delay of 0.1 s.

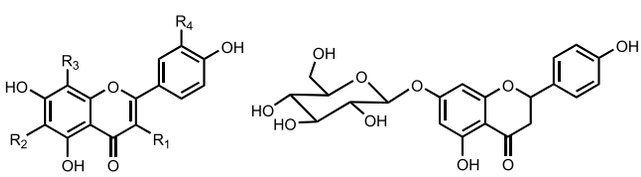
NMR spectroscopy. The NMR experiments on **1–12** were carried out at 600.13 and 150.90 MHz for ^{1}H and ^{13}C, respectively, on a Bruker (Bruker Biospin AG, Fallanden, Switzerland) model DRX-600 instrument equipped with a multinuclear inverse probe for one-dimensional ^{1}H and two-dimensional heteronuclear single quantum coherence (^{1}H-^{13}C HSQC), heteronuclear multiple bond correlation (^{1}H-^{13}C HMBC), double quantum filtered correlation (^{1}H-^{1}H DQF-COSY), total correlation (^{1}H-^{1}H TOCSY) and nuclear Overhauser and exchange (NOESY) spectroscopies. The compensated attached proton test (CAPT) experiment was performed with a ^{1}H/^{13}C BBO probe on a Bruker AM-400 instrument. The temperatures were stabilized at 25°C (cf. Fossen *et al.*, 2003 for further experimental details). The samples were prepared by dissolving individual flavonoids in deuterated methanol (CD_3OD) or deuterated dimethylsulphoxide (DMSO-*d*6) to give the following concentrations: **1** (0.03 M), **2** (0.02 M), **3** (0.14 M), **4** (0.06 M), **5** (0.02 M), **6** (0.04 M), **7** (0.03 M), **8** (0.02 M), **9** (0.03 M), **10** (0.02 M), **11** (0.11 M) and **12** (0.17 M). The residual solvent signals of CD_3OD at δ 3.4 and 49.0 for ^{1}H and ^{13}C, respectively, and of DMSO-*d*6 at δ 2.49 and 39.6 for ^{1}H and ^{13}C, respectively, were used as secondary references. The relative proportions of the two rotamers of each pair were calculated using the integration results of the one-dimensional ^{1}H-NMR spectra.

RESULTS AND DISCUSSION

The HPLC profile of the methanolic extract of dried 'Crataegi folium cum flore' detected at 320 ± 20 nm was very complex. Altogether 12 compounds, (**1–12**; Fig. 1), were separated and purified by a combination of Amberlite XAD-7 column chromatography, Sephadex LH-20 gel filtration and preparative HPLC. Most of these compounds produced two absorption maxima in the regions 255–284 and 328–360 nm, respectively, which are typical for flavonoids. Their structures were elucidated using HPLC-DAD, one- and two-dimensional NMR spectroscopy and electrospray MS (Tables 1–3). Compound **1** is novel, and **4** has previously not been reported to occur in the genus *Crataegus*.

Identification of 8-methoxykaempferol 3-*O*-(6″-malonyl-*β*-glucopyranoside) (1) by homo- and heteronuclear NMR

The UV spectrum of **1** was typical for a flavonol with only one hydroxyl group on its B-ring. The



1-3, 5-12 **4**

	R_1	R_2	R_3	R_4
1	6″-*O*-mal-*O*-*β*-glc	H	OCH_3	H
2	*O*-*β*-glc	H	OCH_3	H
3	*O*-*β*-gal	H	H	OH
5	H	H	*C*-*β*-glc	OH
6	H	*C*-*β*-glc	H	OH
7	H	H	*C*-*β*-glc	H
8	H	*C*-*β*-glc	H	H
9	*O*-robinobiose	H	H	OH
10	H	*C*-neoh	H	H
11	H	H	*C*-neoh	H
12	H	H	4‴-*O*-actyl-*C*-neoh	H

Figure 1. Structures of the flavonoids isolated from methanolic extract of 'Crataegi folium cum flore'. (**1**) 8-Methoxykaempferol 3-*O*-(6″-malonyl-*β*-glucopyranoside), (**2**) 8-methoxykaempferol 3-*O*-*β*-glucopyranoside, (**3**) quercetin 3-*O*-*β*-galactopyranoside, (**4**) naringenin 7-*O*-*β*-glucopyranoside, (**5**) luteolin 8-*C*-*β*-glucopyranoside, (**6**) luteolin 6-*C*-*β*-glucopyranoside, (**7**) apigenin 8-*C*-*β*-glucopyranoside, (**8**) apigenin 6-*C*-*β*-glucopyranoside, (**9**) quercetin 3-*O*-(6″-*O*-*α*-rhamnopyranosyl-*β*-galactopyranoside), (**10**) apigenin 6-*C*-(2″-*O*-*α*-rhamnopyranosyl-*β*-glucopyranoside), (**11**) apigenin 8-*C*-(2″-*O*-*α*-rhamnopyranosyl-*β*-glucopyranoside), and (**12**) apigenin 8-*C*-(2″-*O*-*α*-(4‴-acetyl-rhamnopyranosyl)-*β*-glucopyranoside).

Table 1. Chromatographic (HPLC) and spectral (UV and electrospray MS) data of flavonoids 1–12 isolated from the methanolic extract of 'Crataegi folium cum flore'

Compound[a]	ES-MS, pseudo-molecular ion (*m/z*)	On-line HPLC-DAD		
		λ_{max} (nm)		t_R (min)
1	587 $[M+Na]^+$	356	272	17.70
2	501 $[M+Na]^+$	340	269	16.60
3	465 $[M+H]^+$	358	255	16.54
4	435 $[M+H]^+$	340	284	16.07
5	449 $[M+H]^+$	350	270	14.23
6	449 $[M+H]^+$	350	267	14.72
7	433 $[M+H]^+$	340	269	15.14
8	433 $[M+H]^+$	338	270	16.59
9	611 $[M+H]^+$	356	255	17.77
10	579 $[M+H]^+$	339	270	15.90
11	579 $[M+H]^+$	340	270	14.70
12	621 $[M+H]^+$	340	270	18.34

[a] See Fig. 1 for structures and nomenclature.

one-dimensional ^{1}H-NMR spectrum revealed two semi-doublets (AA′XX′ type) at δ 8.18 ('*d*', 8.9 Hz; H-2′/6′) and δ 6.99 ('*d*', 8.9 Hz; H-3′/5′), a singlet at δ 6.37 (H-6) (Table 2), and a 3H singlet at δ 3.99 (OCH_3) in accordance with 8-methylherbacetin, a kaempferol derivative with its 8-position occupied by a methoxy-group. The NOESY cross-peak between the methyl protons at δ 3.99 and H-2′/6′ at δ 8.18 confirmed that the methoxy group was connected to the 8-position of the aglycone. The corresponding carbon shifts were assigned by the ^{1}H-^{13}C HSQC experiments, while the remaining quaternary carbon shifts were assigned from long-range couplings in the ^{1}H-^{13}C HMBC spectrum (Fig. 2) and the ^{13}C resonances in the CAPT spectrum. The following cross peaks in the HMBC spectrum at δ 8.18/159.33 (H-2′,6′/C-2), δ 5.26/135.33 (H-1″/C-3), δ 6.37/179.57 (H-6/C-4), δ 6.37/157.97 (H-6/C-5), δ 6.37/158.57 (H-6/C-7), δ 6.37/105.59 (H-6/C-10), δ 3.99/129.14 (OCH_3/C-8), and δ 8.18/161.68 (H-2′,6′/C-4′) were of particular importance for assignments of the aglycone substitution pattern (Fig. 2). Furthermore the one-dimensional ^{1}H-NMR spectrum showed a doublet at δ 5.26 (*d*, 7.28 Hz) that, together with six sugar resonances in the CAPT spectrum, were in accordance with a β-glucopyranose unit. The HMBC cross-peak at δ 5.26/135.33 (Fig. 2) confirmed that the sugar moiety was connected to the aglycone 3-position. The downfield shifts of H-6A″ (δ 4.35) and H-6B″ (δ 4.21) revealed 6-substitution of the glucosyl moiety. The HMBC cross-peaks at δ 4.35/168.01 and at δ 4.21/168.01 (Fig. 2) confirmed this substitution. A singlet at δ 3.27 in the ^{1}H-NMR spectrum, which decreased during storage in the deuterated NMR solvent, was typical for CH_2 protons of a malonyl group. Indeed, a molecular ion at *m/z* 587 (corresponding to $[C_{25}H_{24}O_{15}$ + $Na]^+$), and a fragment ion at *m/z* 317 (corresponding to [aglycone + $H]^+$) in the electrospray MS of **1**, were in

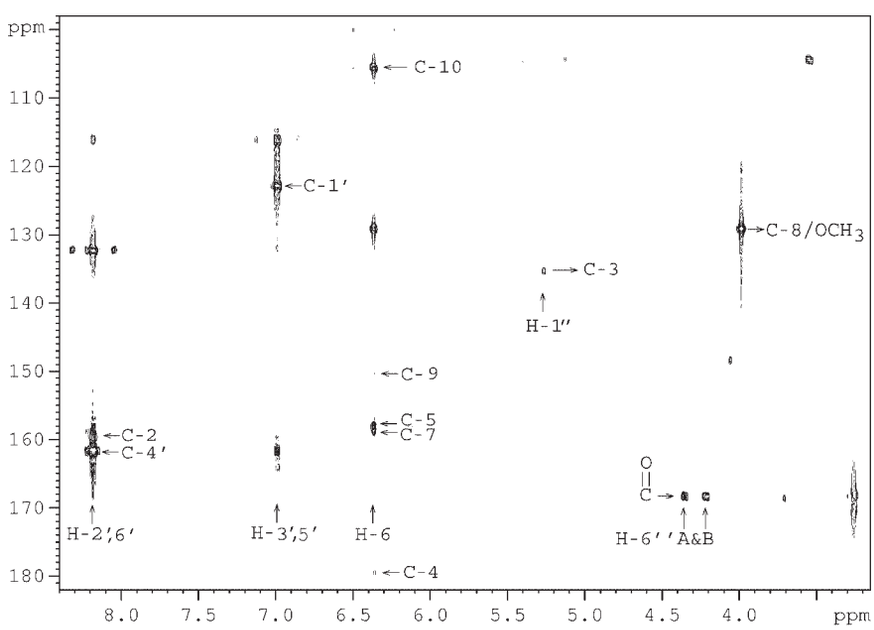


Figure 2. The lower field section of the HMBC spectrum of the novel compound **1** [8-methoxykaempferol 3-*O*-(6″-malonyl-β-glucopyranoside)]. Important assignments of most of the quaternary carbons and the connection points have been labelled.

Table 2. ^{1}H-NMR spectral data (δ in ppm and J in Hz) for the flavonoids 1–12[a] dissolved in CD_3OD or DMSO-*d*6 at 25°C

	1 CD_3OD	**2** CD_3OD	**3** DMSO	**4** CD_3OD	**5_A** CD_3OD[b]	**5_B** CD_3OD[b]	**6** DMSO[b]	**7_A** DMSO[b]	**7_B** DMSO[b]	**8** CD_3OD	**9** CD_3OD	**10** CD_3OD	**10_A** DMSO[b]	**10_B** DMSO[b]	**11_A** CD_3OD[b]	**11_B** CD_3OD[b]	**12_A** CD_3OD[b]	**12_B** CD_3OD[b]
2				5.46 *dd* 2.9, 13.0														
3				3.25[c] *dd* 13.0, 17.3	6.62 *s*	6.63 *s*	6.66 *s*	6.77 *s*	6.79 *s*	6.67 *s*		6.70 *s*	6.76 *s*		6.66 *s*	6.67 *s*	6.68 *s*	6.72 *s*
6	6.37 *s*	6.36 *s*	6.41 *d* 2.0	6.27 *d* 2.2	6.36 *s*			6.26 *s*	6.24 *s*		6.29 *d* 2.1				6.36 *s*	6.35 *s*	6.39 *s*	6.34 *s*
8			6.21 *d* 2.0	6.30 *d* 2.2			6.47 *s*			6.58 *s*	6.48 *d* 2.1	6.62 *s*	6.50 *s*	6.48 *s*				
2′	8.18 ′*d*′ 8.9	8.22 ′*d*′ 9.0	7.54 *d* 2.3	7.40 ′*d*′ 8.6	7.64 *br. s*	7.50 *br. s*	7.39 *d* 2.2	8.01 ′*d*′ 8.6	7.91 ′*d*′ 8.4	7.91 ′*d*′ 8.9	7.96 *d* 2.2	7.94 ′*d*′ 8.8	7.91 ′*d*′ 8.7		8.04 ′*d*′ 8.7	7.89 ′*d*′ 8.7	8.07 ′*d*′ 9.0	7.89 ′*d*′ 9.0
3′	6.99 ′*d*′ 8.9	7.00 ′*d*′ 9.0		6.90 ′*d*′ 8.6				6.88 ′*d*′ 8.6	6.94 ′*d*′ 8.4	7.00 ′*d*′ 8.9		7.02 ′*d*′ 8.8	6.91 ′*d*′ 8.7		7.01 ′*d*′ 8.7	7.00 ′*d*′ 8.7	7.02 ′*d*′ 9.0	7.00 ′*d*′ 9.0
5′	6.99 ′*d*′ 8.9	7.00 ′*d*′ 9.0	6.82 *d* 8.5	6.90 ′*d*′ 8.6	6.99 *d* 8.2		6.88 *d* 8.4	6.88 ′*d*′ 8.6	6.94 ′*d*′ 8.4	7.00 ′*d*′ 8.9	6.96 *d* 8.5	7.02 ′*d*′ 8.8	6.91 ′*d*′ 8.7		7.01 ′*d*′ 8.7	7.00 ′*d*′ 8.7	7.02 ′*d*′ 9.0	7.00 ′*d*′ 9.0
6′	8.18 ′*d*′ 8.9	8.22 ′*d*′ 9.0	7.67 *dd* 8.5, 2.3	7.40 ′*d*′ 8.6	7.60 br.*d* 8.2	7.50 br. *s*	7.41 *dd* 2.2, 8.4	8.01 ′*d*′ 8.6	7.91 ′*d*′ 8.4	7.91 ′*d*′ 8.9	7.68 *dd* 8.5, 2.2	7.94 ′*d*′ 8.8	7.91 ′*d*′ 8.7		8.04 ′*d*′ 8.7	7.89 ′*d*′ 8.7	8.07 ′*d*′ 9.0	7.89 ′*d*′ 9.0
CH_3O	3.99 *s*	3.98 *s*																
β-Glucoside			*β-Galactoside*	*β-Glucoside*														
1″	5.26 *d* 7.3	5.39 *d* 7.6	5.38 *d* 7.7	5.06 *d* 7.2	5.07 *d* 9.7	5.16 *d* 9.7	4.57 *d* 9.7	4.67 *d* 9.8	4.82 *d* 9.8	4.99 *d* 9.9	5.16 *d* 7.8	5.03 *s(b)*	4.61 *d* 9.8	4.66 *d* 9.7	5.12 *d* 9.9	5.21 *d* 9.9	5.13 *d* 9.9	5.24 *d* 9.8
2″	3.56 *m*	3.55 *dd* 9.1, 7.6	3.58 *dd* 7.7, 9.6	3.54 *m*	4.20 *t* 9.5		4.03 *t* 9.4	3.82 *t* 9.6	3.86 *m*	4.26 *t* 9.9	3.92 *dd* 7.8, 9.8	4.36 *m*	4.37 *t* 9.5	4.17 *t* 9.5	4.34 *dd* 9.9, 8.6	4.32 *t* 9.0	4.31 *dd* 9.9, 8.6	4.18 *dd* 9.8, 8.9
3″	3.54 *m*	3.51 *t* 9.1	3.38 *dd* 3.3, 9.6	3.54 *m*	3.62 *m*	3.67 *m*	3.19 *t* 8.6	3.24 *m*	3.31 *m*	3.57 *m*	3.66 *dd* 3.5, 9.8	3.66 *m*	3.32 *m*	3.36 *m*	3.74 *m*	3.78 *m*	3.77 *dd* 8.6, 9.2	3.84 *t* 8.9
4″	3.42 *m*	3.40 *m*	3.66 *d(b)* 3.3	3.48 *t*, 9.4	3.78 *t* 9.2	3.63 *m*	3.11 *t* 9.2	3.37 *t* 9.4	3.24 *m*	3.57 *m*	3.90 *dd* 1.0, 3.5	4.66 *m*	3.12 *m*		3.74 *m*	3.78 *m*	3.72 *t* 9.2	3.65 *m*
5″	3.49 *m*	3.30 *m*	3.34 *dd*, 6.0, 6.3	3.55 *m*	3.56 *m*	3.63 *m*	3.15 *m*	3.21 *m*	3.34 *m*	3.50 *m*	3.74 *t*, 6.3	3.47 *m*	3.16 *m*	3.11 *m*	3.55 *m*	3.64 *m*	3.57 *dd(b)* 5.9, 9.2	3.67 *m*
6A″	4.35 *dd* 2.1, 11.8	3.79 *dd* 2.2, 11.8	3.46 *dd* 6.0, 10.6	3.97*dd* 1.9, 12.2	4.06 *m*	3.99 *m*	3.68 *dd* 1.8, 12.0	3.75 *dd* 1.4, 11.6	3.68 *m*	3.98 *m*	3.50 *dd* 6.8, 10.2	3.97 *m*	3.69 *m*	3.68 *m*	4.06 *m*	4.03 *m*	4.09 *dd* 1.4, 12.3	4.04 *dd* 1.4, 12.3
6B″	4.21 *dd* 5.7, 11.8	3.62 *dd* 5.6, 11.8	3.38 *dd* 6.3, 10.6	3.79 *dd* 5.4, 12.2	3.94 *dd* 5.3, 12.0	3.87 *m*	3.40 *dd* 6.3, 12.0	3.51 *dd* 6.7, 11.6	3.48 *m*	3.84 *m*	3.84 *dd* 5.9, 10.2	3.81 *m*	3.35 *m*	3.41 *m*	3.89 *m*	3.88 *m*	3.91 *m*	3.91 *m*
6″-O-Malonyl																		
2	3.27 *s*																	

Table 2. Continued

	1 CD_3OD	**2** CD_3OD	**3** DMSO	**4** CD_3OD	**5_A** CD_3OD[b]	**5_B** CD_3OD[b]	**6** DMSO[b]	**7_A** DMSO[b]	**7_B** DMSO[b]	**8** CD_3OD	**9** CD_3OD	**10** CD_3OD	**10_A** DMSO[b]	**10_B** DMSO[b]	**11_A** CD_3OD[b]	**11_B** CD_3OD[b]	**12_A** CD_3OD[b]	**12_B** CD_3OD[b]
α-Rhamnosyl																		
1′											4.62 *d* 1.7	5.32 *s(b)*	5.08 *s(b)*	5.02 *s(b)*	5.19 *d* 1.8	5.29 *d* 1.8	5.39 *d* 1.8	5.50 *d* 1.7
2′											3.67 *dd,* 1.7, 3.5	3.96 *dd* 1.8, 3.2	3.59 *m*	3.61 *m*	3.94 *dd* 1.8, 3.2	3.88 *dd* 1.8, 3.2	3.91 *m*	3.80 *dd* 1.7, 3.3
3′											3.60 *dd,* 3.5, 9.6	3.51 *m*	3.12 *m*	3.09 *m*	3.49 *dd* 3.2, 9.5	3.16 *m*	3.57 *dd* 3.3, 9.8	3.10 *dd* 3.3, 9.8
4′											3.38 *t* 9.6	3.21 *t* 9.7	2.91 *m*		3.21 *t* 9.5	3.16 *m*	4.70 *t* 9.8	4.63 *t* 9.8
5′											3.62 *m*	2.64 *m*	2.31 *m*		2.53 *dd* 6.3, 9.5	2.40 *dd* 6.3, 9.5	2.41 *m*	2.17 *m*
6′											1.28 *d* 6.3	0.84 *s(b)*	0.51 *d* 6.1	0.59 *d* 6.1	0.73 *d* 6.3	0.87 *d* 6.3	0.77 *d* 6.3	0.60 *d* 6.3
4‴-O-Acetyl																		
2																	2.11 *s*	2.02 *s*

[a] See Fig. 1 for structures and nomenclature.
[b] Subscripts A and B refer to the major and minor rotamers, respectively.
[c] 3B: 2.83 *dd* 2.9, 17.3. *s* = singlet, *d* = dublet, *t* = triplet, *m* = multiplet, *b* = broad.

Table 3. ^{13}C-NMR spectral data (δ in ppm) for the flavonoids 1–12[a] dissolved in CD_3OD or DMSO-*d*6 at 25°C

	1 CD_3OD	**2** CD_3OD	**3** DMSO	**4** CD_3OD	**5_A** CD_3OD[b]	**5_B** CD_3OD[b]	**6** DMSO[b]	**7_A** DMSO[b]	**7_B** DMSO[b]	**8** CD_3OD	**9** CD_3OD	**10** CD_3OD	**10_A** DMSO[b]	**10_B** DMSO[b]	**11_A** CD_3OD[b]	**11_B** CD_3OD[b]	**12_A** CD_3OD[b]	**12_B** CD_3OD[b]
2	159.33	158.88	156.44	80.62	166.70	166.60	163.71	164.02	163.81	166.17	158.92	166.21	163.21		166.53	165.74	166.66	165.95
3	135.33	135.50	133.69	44.13	103.68	103.59	102.87	102.52	102.72	103.88	135.86	103.92	102.76		103.53	103.46	103.58	104.21
4	179.57	179.73	177.64	198.52	184.16		181.93	182.18	181.53	184.03	179.45	184.15	181.38	182.53	183.99		184.11	
5	157.97	150.42	156.50	159.07	162.66	162.85	160.76	160.46	160.79	162.02	162.92	164.60	161.32	159.97	162.56	162.47	162.82	163.20
6	100.14	100.10	93.71	97.99	99.37	100.63	108.95	98.20	99.46	109.16	99.95	109.56	108.96	108.68	99.81	101.06	99.77	101.32
7	158.57	158.47	164.21	167.00	164.57	164.62	163.35	162.63	162.96	164.84	166.08	164.76	162.64	163.46	164.04	164.35	164.12	164.34
8	129.14	129.13	98.81	96.92	105.78	105.81	93.57	104.68	104.57	95.24	94.82	95.08	92.96	94.29	105.52	105.41	105.75	105.61
9	150.39	158.05	161.42	164.93	158.11	158.03	156.26	156.06	156.03	158.70	158.43	158.76	156.23	156.43	157.77	156.60	157.80	156.34
10	105.59	105.74	104.13	104.91	105.15	105.11	103.46	104.12	103.89	105.21	105.54	105.45	103.85	103.49	105.89	105.86	105.98	104.74
1′	122.80	122.91	121.33	130.85	124.05		121.49	121.68	121.62	123.09	122.78	123.14	121.08		123.42	123.35	123.36	123.32
2′	132.30	132.27	116.14	129.09	114.99	114.17	113.38	129.05	128.57	129.43	117.96	129.46	128.40		129.99		130.13	129.52
3′	116.15	116.19	144.90	116.33	147.07		145.82	115.87	116.10	117.02	145.72	117.04	116.06		116.91		117.01	117.15
4′	161.68	161.70	148.53	164.59	150.95		149.78	161.22	161.35	162.77	149.99	162.81	161.15		162.56	162.47	162.79	
5′	116.15	116.19	115.35	116.33	116.73	116.45	116.12	115.87	116.10	117.02	116.10	117.04	116.06		116.91		117.01	117.15
6′	132.30	132.27	122.19	129.09	120.92	120.60	119.04	129.05	128.57	129.43	123.00	129.46	128.40		129.99		130.13	129.52
8-OMe	62.01	61.96																
	β-Glucoside		*β-Galactoside*	*β-Glucoside*														
1″	104.36	104.07	102.00	101.24	75.36	76.28	73.12	73.45	74.35	75.28	105.98	73.53	71.35	71.25	73.59	74.86	73.77	75.30
2″	75.61	75.74	71.38	74.64	72.85	72.68	70.27	70.89	71.09	72.60	73.14	77.52	74.61	75.80	78.04	77.88	76.09	76.29
3″	77.83	78.05	73.30	77.79	80.33	79.84	79.02	78.70	78.72	80.12	75.08	81.37	80.00	79.67	81.48	81.17	81.80	81.45
4″	71.15	71.40	68.11	71.14	72.30	71.29	70.70	70.25	70.60	71.79	70.18	77.19	70.92	70.92	72.12	71.56	72.51	71.50
5″	75.53	78.45	75.99	78.24	82.93	82.62	81.65	81.74	81.93	82.62	75.28	82.42	81.42	81.52	82.68	82.75	82.99	83.00
6″	64.89	62.68	60.33	62.33	63.23	63.51	61.57	61.27	61.35	62.86	67.32	62.90	61.78	61.32	63.01	62.55	63.13	62.45
6″-O-Malonyl																		
1	168.25																	
2	41.82																	
3	169.98																	
α-Rhamnosyl																		
1‴											101.90	102.44	100.41	100.70	102.37		101.15	101.19
2‴											72.04	72.35	70.58	70.62	72.37	72.02	72.09	71.80
3‴											72.27	72.05	70.95	70.35	71.84	71.88	70.02	
4‴											73.87	73.59	71.61		73.43	73.14	75.16	
5‴											69.71	69.88	68.29		69.84	69.91	67.27	67.03
6‴											17.97	18.02	17.74	17.66	17.97	17.90	17.84	17.95
4‴-O-Acetyl																		
1																	172.54	
2																	21.00	21.03

[a] See Fig. 1 for structures and nomenclature.
[b] Subscripts A and B refer to the major and minor rotamers, respectively.

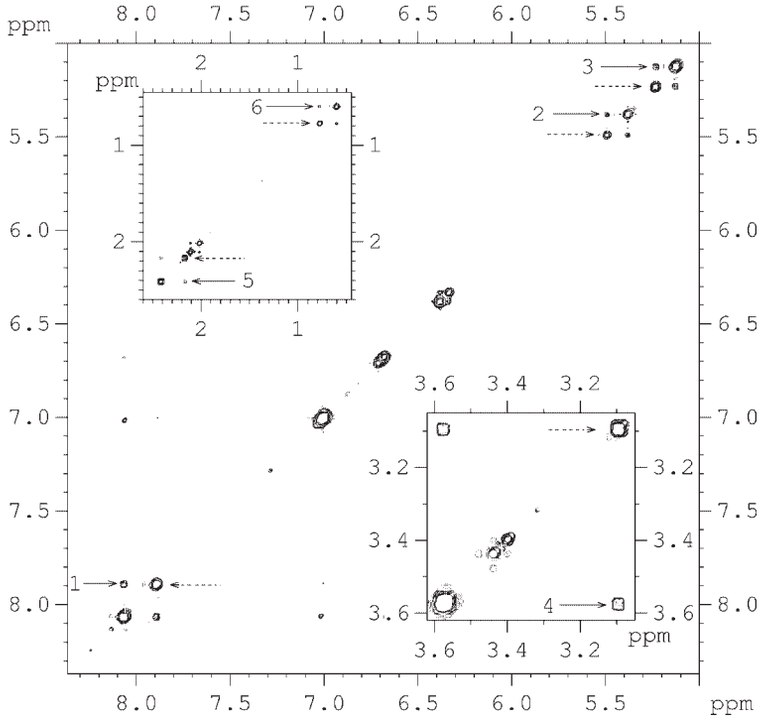


Figure 3. The lower field section of the two-dimensional nuclear Overhauser and exchange spectrum of **12** (in CD_3OD) revealing the exchange cross-peaks between proton signals of the two rotamers, **12A** and **12B**. Two higher-field regions have been included. Arrows labelled **1–6** indicate the exchange cross-peaks between protons within the following pairs: H-2′,6′A/H-2′,6′B, H-1′A/H-1′B, H-1″A/H-1″B, H-3′A/H-3′B, H-5′A/H-5′B and H-6′A/H-6′B, respectively. Dashed arrows indicate the diagonal peaks of the minor rotamer.

agreement with the novel flavonol 8-methoxykaempferol 3-*O*-(6″-malonyl-*β*-glucopyranoside).

NMR analysis of the rotamers of flavone *C*-glycosides

Tables 2 and 3 show that luteolin 8-*C*-glucoside (**5**), apigenin 8-*C*-glucoside (**7**), apigenin 6-*C*-neohesperidoside (**10**), apigenin 8-*C*-neohesperidoside (**11**) and apigenin 8-*C*-(4‴-acetylneohesperidoside) (**12**) exhibit most of their ^{1}H- and ^{13}C-NMR signals (Tables 2 and 3) in duplicated sets. These signals reveal two conformational isomers created by rotational hindrance at the $C(sp^3)$–$C(sp^2)$ glucosyl–flavone linkage in each of these 6-*C* or 8-*C* substituted flavones. The equilibrium between the two rotamers was supported by observations of strong exchange peaks between equivalent protons of each rotameric pairs in their NOESY spectra (Fig. 3). The rotamers of **5**, **11** and **12** were observed in both CD_3OD and DMSO-*d*6 solutions (Table 4). Rotamers of **7** and **10** were only observed in DMSO-*d*6, but the former was not soluble in CD_3OD. The impact of temperature on the rotational equilibrium of some flavone *C*-glycosides has been reported by several authors (Davoust *et al.*, 1980; Nørbæk *et al.*, 2000; Zhang *et al.*, 2003).

The presence of rotamers leading to signal doubling in the NMR spectra has been discovered for flavones containing an 8-*C*-hexosyl (sugar hexose) substituent (Markham *et al.*, 1987; Jay, 1993). Rotamers for

Table 4. Relative proportions of the major and minor rotamers of luteolin 8-*C*-glucoside (5), apigenin 8-*C*-glucoside (7), apigenin 6-*C*-neohesperidoside (10), apigenin 8-*C*-neohesperidoside (11) and apigenin 8-*C*-(4‴-acetylneohesperidoside) (12) in DMSO-*d*6 and CD_3OD at 25°C

	DMSO-*d6*		CD_3OD	
Flavone	Major rotamer	Minor rotamer	Major rotamer	Minor rotamer
5	1.00	0.21	1.00	0.44
7	1.00	0.15	[a]	[a]
10	1.00	0.88	[b]	[b]
11	1.00	0.06	1.00	0.26
12	1.00	0.09	1.00	0.58

[a] Insoluble in methanol.
[b] No distinct rotameric signals detected; however, broadening of several signals in the sugar region was observed.

analogous pigments with 6-*C*-hexosyl sugar substituents were not observed. As an explanation, interaction between the flavone B-ring and the 8-*C*-hexosyl substituent leading to restricted rotation of the B-ring and/or the hexose has been suggested to give rise to a mixture of two NMR-distinguishable rotamers (Markham *et al.*, 1987). Indeed, the 8-*C*-monoglucosides of apigenin and luteolin, **5** and **7**, respectively, exhibited signal duplication in their NMR spectra recorded in DMSO-*d*6 (Tables 2 and 3), while the corresponding spectra of the 6-*C*-glucosides of apigenin and luteolin (**6** and **8**), in which the *C*-glycosyl residues were relatively distant from the flavone B-rings, did not exhibit signal duplication and thus no rotameric conformers.

Rotameric conformers of flavone 6-*C*-glycosides have been observed (Davoust *et al.*, 1980; Cheng *et al.*, 2000; Lewis *et al.*, 2000; Nørbæk *et al.*, 2000). Davoust *et al.* (1980) reported that only 6-*C*-*β*-glucosylflavones substituted by a methoxyl or an *O*-*β*-glucosyl group in the 7 position gave rise to split signals. This was explained by steric hindrance by the 7-substituents in the *ortho*-position to 6-*C*-glucosyl (Davoust *et al.*, 1980), in accordance with the later reports concerning rotamers of 6-*C*-glucosylflavones. Contrary to this, apigenin 6-*C*-neohesperidoside (**10**), which lacks a 7-methoxyl/7-*O*-glycosyl substitution, showed two rotamers when dissolved in DMSO-*d*6 (Table 4). Thus, restricted rotation around the $C(sp^3)$–$C(sp^2)$ glucosyl–flavone linkage might also be observed in some flavone 6-*C*-glycosides with bulky glycosyl substituents. Based on conformational analysis, Cheng *et al.* (2000) excluded the existence of rotameric conformers of apigenin 6-*C*-(2″-*O*-glucosylglucoside) at room temperature because of the low-energy barrier between the two potential energy minima. For the *C*-8 substituted flavone *C*-glucosides (**5**, **7**, **11** and **12**) most of the aromatic and sugar signals were duplicated, whereas for apigenin 6-*C*-neohesperidoside (**10**) only H-8 and some of the sugar signals were duplicated (Tables 2 and 3).

When introducing a second sugar unit (rhamnosyl) at the 8-*C*-glucosyl 2″-position (**11**) and an additional acetyl group at the rhamnose 4′-hydroxyl (**12**) of apigenin 8-*C*-glucoside (both dissolved in DMSO-*d*6), the relative proportions of the minor rotamers were 0.06 and 0.09, respectively (Table 4). Markham *et al.* (1987) reported no rotamers for similar flavone 8-*C*-glycosides. This indicates the strength of using two-dimensional NOESY for the detection of rotational equilibrium forms. The relative low proportions of the minor rotamers of **5**, **11** and **12** increased considerably when these compounds were dissolved in CD_3OD (Table 4), which indicated a lower energy barrier in the equilibrium between the two rotamers in CD_3OD compared with DMSO-*d*6.

Acknowledgements

The authors wish to thank the Norwegian Research Council (NFR) for support. T.F. and S.R. gratefully acknowledge NFR and the State Education Loan Fund, respectively, for their fellowships.

REFERENCES

Al Makdessi S, Sweidan H, Dietz K, Jacob R. 1999. Protective effect of *Crataegus oxyacantha* against reperfusion arrhythmias after global no-flow ischemia in the rat heart. *Basic Res Cardiol* **94**: 71–77.

Ammon HPT, Kaul R. 1994. *Crataegus.* Herz-Kreislauf-Wirkungen von *Crataegus* Extrakten, Flavonoiden und Procyanidinen. *Deutsche Apotheker Zeitung* **134**: 2521–2535.

Andersen ØM. 1988. Semipreparative isolation and structure determination of pelargonidin 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside and other anthocyanins from the tree *Dacrycarpus dacrydioides*. *Acta Chem Scand* **42**: 462–468.

Cheng G, Bai Y, Zhao Y, Tao J, Liu Y, Tu G, Ma L, Liao N, Xu X. 2000. Flavonoids from *Ziziphus jujube Mill* var. *spinosa*. *Tetrahedron* **56**: 8915–8920.

Davoust D, Massias M, Molho D. 1980. ^{13}C-NMR investigation of flavonoid *C*-*β*-D-glucosides. Detection of a conformational equilibrium. *Org Magn Reson* **13**: 218–219.

German Commission. 1994. Monograph: Crataegi folium cum flore, *Bundesanzeiger*, no. 133.

Fossen T, Slimestad R, Andersen ØM. 2003. Anthocyanins with 4′-glucosidation from red onion, *Allium cepa*. *Phytochemistry* **64**: 1367–1374.

Jay M. 1993. *C*-glucosylflavonoids. In *The Flavonoids: Advances in Research since 1986*, Harborne JB (ed.). Chapman and Hall: New York; 57–93.

Kumazawa T, Kimura T, Matsuba S, Sato S, Onodera J. 2001. Synthesis of 8-*C*-glucosylflavones. *Carbohyd Res* **334**: 183–193.

Lewis KC, Maxwell AR, McLean S, Reynolds WF, Enriquez RG. 2000. Room temperature (^{1}H and ^{13}C) and variable-temperature (^{1}H) NMR studies on spinosin. *Magn Reson Chem* **38**: 771–774.

Markham KR, Mues R, Stoll M, Zinsmeister HD. 1987. NMR-spectra of flavone di-*C*-glycosides from *Apometzgeria pubescens* and the detection of rotational-isomerism in 8-*C*-hexosylflavones. *Zeitschr Naturforsch* **42**C: 1039–1042.

Melikoglu G, Mericli AH. 2000. Flavonoids of *Crataegus stevenii*. *Pharmazie* **55**: 326–327.

Nørbæk R, Brandt K, Kondo T. 2000. Identification of flavone *C*-glycosides including a new flavonoid chromophore from barley leaves (*Hordeum vulgare* L.) by improved NMR techniques. *J Agric Food Chem* **48**: 1703–1707.

Schuessler M, Hoelzl J, Fricke U. 1995. Myocardial effects of flavonoids from Crataegus species. *Arznei Forsch* **45**: 842–845.

Shahat AA, Cos P, de Bruyne T, Apers S, Hammouda FM, Ismail SI, Azzam S, Claeys M, Goovaerts E, Pieters L, Vanden Berghe D, Vlietinck AJ. 2002. Anti-viral and anti-oxidant activity of flavonoids and proanthocyanidins from *Crataegus sinaica*. *Planta Med* **68**: 539–541.

Sticher O, Meier B. 1998. Hawthorn (*Crataegus*): biological activity and new strategies for quality control. In: *ACS Symposium Series 691: Phytomedicines of Europe*. American Chemical Society: New York; 241–262.

Weinges K, Marx HD, Goritz K. 1970. Contributions to proanthocyanins. 15. Rotational hindrance at $C(sp^2)$-$C(sp^3)$ linkage of 4-aryl-substituted polymethoxyflavans. *Chem Beri-Recu* **103**: 2336–2343.

Zhang PC, Xu SX. 2003. *C*-Glucoside flavonoids from the leaves of *Crataegus pinnatifida* BGE. var. *major* N.E.BR. *J Asian Nat Prod Res* **5**: 131–136.

Zhang PC, Wang YH, Liu X, Yi X, Chen RY, Yu DQ. 2003. Conformational study on 8-*C*-glucosyl-prunetin by dynamic NMR spectroscopy. *Acta Chim Sin* **61**: 1157–1160.