Spectroscopic Characterization of Crocetin Derivatives from *Crocus sativus* and *Gardenia jasminoides*

Marie-Rose Van Calsteren,*,† Martine C. Bissonnette,†,‡ François Cormier,† Christiane Dufresne,† Takahito Ichi,§ J. C. Yves LeBlanc,|| Daniel Perreault,† and Ingeborg Roewer†

Food Research and Development Centre, Agriculture and Agri-food Canada, Saint-Hyacinthe, Quebec, Canada J2S 8E3, San-Ei Gen F.F.I., Inc., Sanwa-cho, Toyonaka, Osaka 561, Japan, and Institute for Environmental Chemistry, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

Crocetin derivatives were extracted and purified from saffron stigmas and gardenia fruits by reversed-phase HPLC. They were characterized by 1D and 2D ^{1}H and ^{13}C NMR and UV−vis spectroscopies and mass spectrometry. The following compounds were found in saffron: *all-trans*-crocetin di(β-D-gentiobiosyl) ester **1a**, *all-trans*-crocetin β-D-gentiobiosyl-β-D-glucosyl ester **1b**, *all-trans*-crocetin di(β-D-glucosyl) ester **1c**, and *all-trans*-crocetin mono(β-D-gentiobiosyl) ester **1d**, as well as 13-*cis*-crocetin di(β-D-gentiobiosyl) ester **2a** and 13-*cis*-crocetin β-D-gentiobiosyl-β-D-glucosyl ester **2b**. Compounds **1a**, **1b**, and **1d** were also present in gardenia, in addition to *all-trans*-crocetin mono(β-D-glucosyl) ester **1e**.

Keywords: *Saffron; gardenia; Crocus sativus; Gardenia jasminoides; crocetin derivatives; carotenoid pigments; mass spectrometry; UV−vis spectroscopy; NMR spectroscopy*

INTRODUCTION

The principal coloring power of saffron (dried stigmas of *Crocus sativus* L.) and gardenia (fruits of *Gardenia jasminoides*) is crocin, a digentiobiosyl derivative of crocetin. Crocin is one of the few carotenoids found in nature that is freely soluble in water, which is the reason for its great application as a food colorant (Timberlake and Henry, 1986). Six glycosides of crocetin have been isolated from saffron and characterized by mass spectrometry and UV−vis spectroscopy (Pfander and Rychener, 1982) and seven from gardenia (Ichi et al., 1995). Pfander and Wittwer (1975a,b) reported the UV−vis, mass, ^{1}H NMR, and IR spectra of the peracetylated derivatives of five *trans*-crocetin glycosides from saffron. Recently, Pfister et al. (1996) have reported isolation and structure elucidation of the peracetylated derivatives of three crocetin glycosides from gardenia and one from saffron. Tarantilis et al. (1995) have used high-performance liquid chromatography with UV−vis photodiode array detection coupled to mass spectrometry to separate and characterize crocetin glycosides carrying one up to five glucoses and differentiate their *trans* and *cis* isomers.

The objective of this study was to further characterize and confirm the structure of crocetin glycosides isolated and purified from saffron and gardenia using spectroscopic techniques (MS, UV−vis, and NMR).

EXPERIMENTAL PROCEDURES

Material. Crocetin was obtained as the pyridine salt from Sigma-Aldrich Canada, Ltd. (Mississauga, ON). The crystals were washed with CD_3OD/C_6D_6 (4:1) to remove pyridine prior to NMR analysis. Dried saffron, "Best Mancha" quality, produced in 1990 in the Gatinais region of France, was purchased from Roidel, Inc. (Louisville, PQ) and stored in darkness. Seven-month-old fruits of *Gardenia jasminoides* having reached full ripeness were collected from greenhouse-grown plants (San-Ei Gen F.F.I., Inc., Toyonaka, Japan).

Purification of Saffron Pigments. Ten batches of powdered stigmas (1.5−1.8 g) were each extracted with 12−15 mL of 50% ethanol at 40 °C for 1 h. Each extract solution was filtered and a 2-mL aliquot applied onto a Sephadex LH-20 column (5 cm i.d. × 45 cm length) previously equilibrated with 50% ethanol. The column was isocratically eluted at a rate of 2 mL/min with 50% ethanol overnight, yielding three intensely pigmented fractions. Fractions of equal composition were pooled and concentrated under vacuum, filtered, and submitted to preparative HPLC. Each time, 2 mL of extract was injected on a Whatman Partisil 10 ODS-2 C_{18} column (20 mm i.d. × 250 mm length) and eluted at a flow rate of 6 mL/min with the following mobile phase: 5% methanol in water was maintained for 2 min, increased linearly to 52.5% methanol over a 10-min period and then to 100% methanol over a 30-min period. The absorbance of the eluate was monitored at 440 nm. Fractions of the eluate corresponding to one peak were collected, concentrated, and reinjected until sufficiently pure.

Purification of Gardenia Pigments. The gardenia fruits were extracted in the cold by triturating in 50% acetone and filtering under vacuum. The residues were re-extracted in the same manner with increasing concentrations of acetone up to 100%. The filtrates were pooled, evaporated to dryness using a rotary evaporator at 40 °C, and dissolved in methanol. The extract was dissolved in 5 equiv of water and applied onto a Diaion HP-20 porous resin column (5 cm i.d. × 20 cm length). The column was rinsed with water, and the pigments were eluted with 80% methanol. The eluate was evaporated to dryness under reduced pressure, dissolved in 100% methanol, and applied onto a Sephadex LH-20 column (4 cm i.d. × 30 cm length). The pigments were eluted with 100% methanol and collected in four separate fractions. Each fraction was further purified by preparative HPLC using a Waters PREP 4000 with a diode array detector set at 420 and 254 nm. The separation was performed on a Nucleosil 5C_{18} (20 mm i.d. × 250 mm length). The mobile phase delivered at a flow rate of 10 mL/min was 50% methanol for the first 15 min, then

* Author to whom correspondence should be addressed [fax (514) 773-8461; e-mail vancalsteren@em.agr.ca].

† Agriculture and Agri-food Canada.

‡ Present address: Transport Canada, CANUTEC, Ottawa, ON, Canada K1A 0N5.

§ San-Ei Gen F.F.I., Inc.

|| National Research Council of Canada.

S0021-8561(96)00348-2 CCC: $14.00

increased linearly to 100% methanol over 15 min, and maintained at 100% methanol for 10 min.

Mass Spectrometry. The mass spectrometry analysis of each saffron pigment was performed using the electrospray technique on a Sciex Instrument Model TAGA 6000E. The potential applied to the electrospray probe varied from 3.20 to 3.40 kV, and the acquisition took place in the positive ion mode. Each sample was dissolved in a distilled water/methanol/ammonia (19:19:2) solution containing 100 mM ammonium acetate. The addition of ammonium acetate favors the formation of an adduct between the compounds of interest and the NH_4^+ ion. Therefore, all masses obtained correspond to $[M + NH_4 + H]^+$.

Identification of gardenia peaks was performed using a VG Biotech desktop-type liquid chromatograph mass spectrometer (LC-MS). The HPLC conditions were as for the purification above, and detection was in the negative ion mode with the electrospray ionization method.

UV−Vis Spectroscopy. UV−vis spectra were obtained in 50% ethanol on a Beckman DU-7 spectrophotometer. They were scanned from 200 to 600 nm at 600 nm/min.

NMR Spectroscopy. ^{1}H NMR spectra were recorded at 25 °C both in a CD_3OD/C_6D_6 (4:1) solvent mixture and in DMSO-d_6 and ^{13}C NMR spectra in DMSO-d_6 on a Bruker AM-300 spectrometer (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C) equipped with a 5-mm ^{1}H/^{19}F probe for ^{1}H spectra and a 10-mm broadband probe for ^{13}C and double-resonance experiments. When the HOD resonance was large compared to the protons of interest, presaturation was used. Connectivities between protons were obtained using 2D COSY (Aue et al., 1976; Bax and Freeman, 1981) with or without presaturation. All normal-mode ^{13}C spectra were acquired with composite-pulse decoupling (WALTZ-16) (Shaka et al., 1983). DEPT spectra (Doddrell et al., 1982) were obtained using a reading pulse of 135°. The 2D HETCORR (Bax and Morris, 1981) or the CHORTLE experiment (Pearson, 1985) was performed using the same basic acquisition parameters as for the DEPT experiment. The 2D COLOC experiment (Kessler et al., 1984) was used to detect long-range connectivities. Alternatively, heteronuclear correlations were obtained with inverse-mode detection (Bendall et al., 1983; Bax et al., 1983). The HMQC experiment was acquired with ^{13}C decoupling using the GARP pulse sequence (Shaka et al., 1985). The HMBC pulse sequence includes a low-pass *J* filter (Bax and Summers, 1986) to suppress one-bond correlations. A Bruker AM-500 spectrometer (500.13 MHz for ^{1}H) equipped with a 5-mm ^{1}H/^{13}C probe was also used for 1D HOHAHA experiments (Davis and Bax, 1985). ^{1}H spectra are referenced to residual benzene at 7.15 ppm or DMSO at 2.49 ppm and ^{13}C spectra to residual DMSO at 39.5 ppm. Simulations of the resolution-enhanced spectra were performed with the Bruker program PANIC and with the program gNMR (Cherwell Scientific). Precision on parameters is reflected by the number of significant digits reported. Coupling constants smaller than the line width are not shown. A modification of the program CHFIT from the ABACUS program library was used for processing data from the CHORTLE experiment.

RESULTS AND DISCUSSION

Six glycosides could be isolated from saffron in sufficient amounts for structure determination. In the order of elution these are **1a**, **1b**, **1c**, **2a**, **2b**, and **1d** (Figure 1). From gardenia, it was possible to obtain four glycosides. In the same order they are **1a**, **1b**, **1d**, and **1e** (Figure 1).

Tables 1 and 2 present the mass spectrometry data for the saffron and gardenia carotenoids, respectively. Molecular ions of each crocetin derivative as an adduct with NH_4^+ in positive ion mode give an *m/z* 20 mass units higher than that of the same derivative in negative ion mode only. The mass of the molecular ion is characteristic of the total number of glucose units but not of their distribution on the aglycon. Other major ions correspond to fragments with fewer glucose units or to the aglycon moiety.

UV−vis data for saffron and gardenia are displayed in Table 3. The exact maximum frequency of the two more intense absorption peaks is not char-

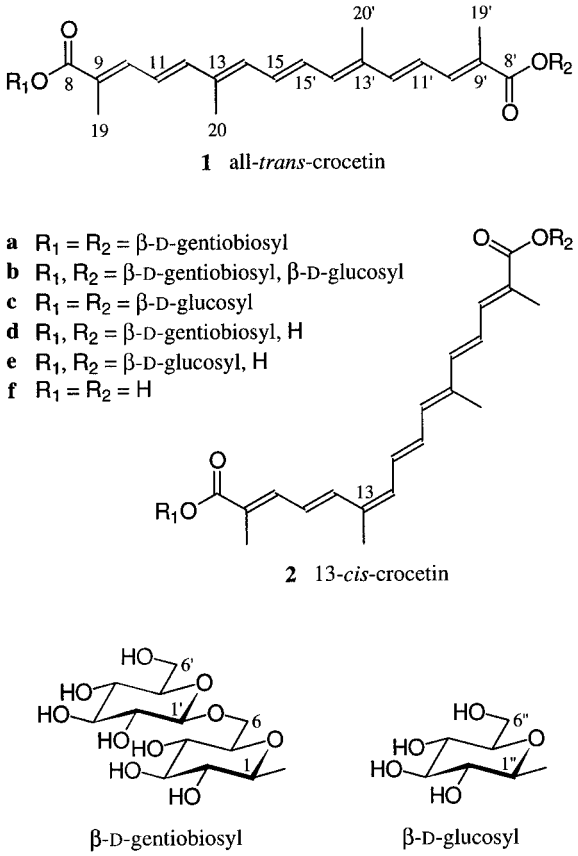

Figure 1. Structures of the crocetin derivatives.

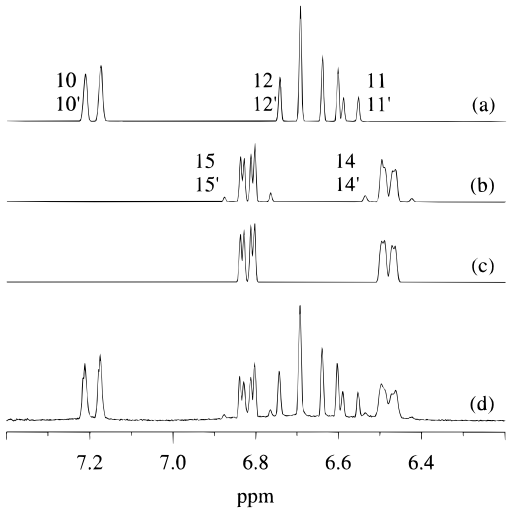

Figure 2. Olefinic region of the resolution-enhanced ^{1}H NMR spectrum of crocetin in DMSO-d_6: (a) simulation of the 19−10−11−12 (or 19′−10′−11′−12′) spin system using the parameters $\delta_{19} = \delta_{19'} = 1.903$, $\delta_{10} = \delta_{10'} = 7.194$, $\delta_{11} = \delta_{11'} = 6.603$, $\delta_{12} = \delta_{12'} = 6.713$, $J_{19-10} = J_{19'-10'} = 1.0$ Hz, $J_{10-11} = J_{10'-11'} = 11.6$ Hz, $J_{11-12} = J_{11'-12'} = 15.0$ Hz; (b) simulation of the 20−14−15−15′−14′−20′ spin system using the parameters $\delta_{20} = \delta_{20'} = 1.962$, $\delta_{14} = \delta_{14'} = 6.481$, $\delta_{15} = \delta_{15'} = 6.820$, $J_{20-14} = J_{20'-14'} = 0.9$ Hz, $J_{14-15} = J_{14'-15'} = 11.5$ Hz, $J_{14-15'} = J_{14'-15} = -1.3$ Hz, $J_{15-15'} = 14.4$ Hz; (c) simulation of the 20−14−15−15′−14′−20′ spin system using the parameters $\delta_{20} = \delta_{20'} = 1.962$, $\delta_{14} = \delta_{14'} = 6.481$, $\delta_{15} = \delta_{15'} = 6.821$, $J_{20-14} = J_{20'-14'} = 0.9$ Hz, $J_{14-15} = J_{14'-15'} = 7.6$ Hz, $J_{14-15'} = J_{14'-15} = 2.7$ Hz; (d) experimental spectrum.

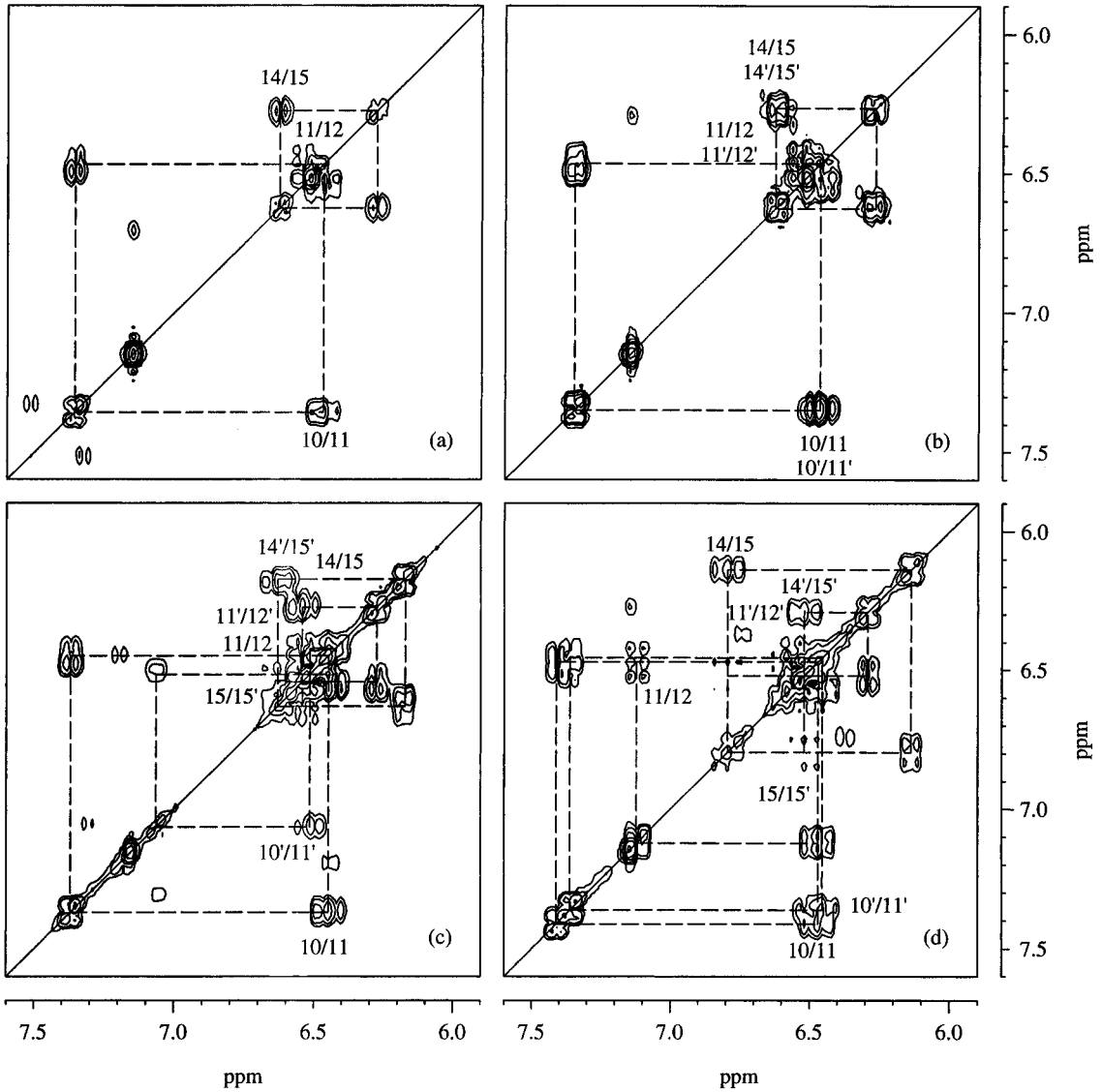

Figure 3. Olefinic region of the ^{1}H NMR two-dimensional COSY spectrum of crocetin derivatives in CD_3OD/C_6D_6 (4:1): (a) *all-trans*-crocetin di(*β*-D-gentiobiosyl) ester **1a**; (b) *all-trans*-crocetin *β*-D-gentiobiosyl-*β*-D-glucosyl ester **1b**; (c) *all-trans*-crocetin mono(*β*-D-gentiobiosyl) ester **1d**; (d) 13-*cis*-crocetin *β*-D-gentiobiosyl-*β*-D-glucosyl ester **2b**. A nonshifted sine window function was employed in both dimensions.

Table 1. Electrospray Mass Spectrometry Data for Saffron Carotenoids[a]

compd	$[M + NH_4 + H]^+$	no. of glucose moieties
1a	995	4
1b	833	3
1c	671	2
1d	671	2
2a	995	4
2b	833	3

[a] Conditions: positive mode, 100 mM CH_3COONH_4 in H_2O/CH_3OH/NH_4OH (19:19:2).

Table 2. Liquid Chromatography Electrospray Ionization Mass Spectrometry Data for Gardenia Carotenoids[a]

compd	$[M - H]^-$	other major ions (*m*/*z*)	no. of glucose moieties
1a	975 (11)[b]	651 (100), 327 (100)	4
1b	813 (15)	489 (45), 327 (100)	3
1d	651 (90)	489 (5), 327 (100)	2
1e	489 (100)	327 (100)	1

[a] Conditions: negative mode. [b] Relative intensities in parentheses.

acteristic of a particular substitution pattern. For compounds **2a** and **2b** from saffron, the spectra show the presence of a peak at 326.5 nm, the so-called "*cis*" peak (Connors et al., 1993), indicating that one of the double bonds along the chain is *cis*. Frequencies of the two major peaks decrease when the aglycon is not symmetric (monosubstituted or *cis*).

In NMR spectroscopy, protons 10 and 14 are easily identified on the COSY spectrum from their long-range coupling across a double bond, respectively to methyls 19 and 20. The olefinic region of the ^{1}H NMR spectral simulations of the two spin systems of crocetin **1f**, which served as a model compound, is shown in Figure 2. It should be noted that, even though the apparent coupling constants for the 20−14−15−15′−14′−20′ spin system are 7.6 (J_{14-15} and $J_{14'-15'}$) and 2.7 Hz ($J_{14-15'}$ and $J_{14'-15}$) as reported in the literature for *all-trans*-crocetin derivatives (Speranza et al., 1984; Ichi et al., 1995), these values have no physical significance and the spectrum is more accurately simulated (presence of non-first-order side peaks) with the values 11.5 (J_{14-15} and $J_{14'-15'}$) and 14.4 Hz ($J_{15-15'}$), together with a long-range coupling of −1.3 Hz ($J_{14-15'}$ and $J_{14'-15}$) (Table 4), the 14-Hz value

Table 3. UV−Vis Spectroscopy Data for Saffron and Gardenia Carotenoids in 50% Ethanol

compd	λ_1 (nm)	λ_2 (nm)	λ_3 (nm)
	Saffron		
1a	440.0	465.5	
1b	440.0	465.5	
1c	438.0	461.0	
1d	433.5	456.0	326.5
2a	435.0	459.0	326.5
2b	435.0	457.5	
	Gardenia		
1a	439.5	464.5	
1b	439.0	464.0	
1d	431.0	453.0	
1e	431.0	453.0	

Table 4. ^{1}H NMR (300.13 MHz) Data for *all-trans*-Crocetin 1f in DMSO-d_6 at 25 °C

proton	δ	multiplicity[a]	J (Hz)	no. of protons
10, 10′	7.194	br d	11.6, 1.0	2H
11, 11′	6.603	dd	15.0, 11.6	2H
12, 12′	6.713	d	15.0	2H
14, 14′	6.481	br ∼dd	11.5, −1.3, 0.9	2H
15, 15′	6.820	∼dd	14.4, 11.5, −1.3	2H
19, 19′	1.903	br s	1.0	6H
20, 20′	1.962	br s	0.9	6H

[a] Abbreviations: s, singlet; d, doublet; br, broad; ∼, first-order approximation.

being characteristic of a *trans* configuration for the 15−15′ double bond. Broad lines were obtained in the ^{1}H spectra of crocetin derivatives in DMSO-d_6, and therefore simulations were only partially possible. For this reason, a mixture of deuterated methanol and benzene (4:1) was used to better resolve the lines. This solvent mixture proved also useful to better spread the sugar chemical shifts. When the aglycon is not symmetric due to a nonesterified carboxylic acid function (as in **1d** or **1e**) or a *cis* double bond (as in **2a** or **2b**) on one side of the molecule, the 19−10−11−12 is different from the 19′−10′−11′−12′ spin system, and protons 20, 14, and 15 are different from 20′, 14′, and 15′, whereas two different sugars on each side of the aglycon have only minor effects (Figure 3). Figure 4 shows the results of the 1D HOHAHA experiment on the olefinic protons of compound **1d**. The ^{1}H NMR data for the aglycon portion of the crocetin derivatives are summarized in Table 5. The absence of an esterified sugar on one side of the aglycon causes a broadening and an upfield shift of 0.28 ppm for proton 10′. For compounds in which a *cis* double bond is present, an upfield shift of 0.14 ppm for proton 14 and downfield shifts of 0.59 and 0.18 ppm for protons 12 and 15, respectively, allow the positioning of the *cis* double bond between carbons 13 and 14.

The anomeric proton of the glucose unit involved in an ester linkage with the crocetin is found around 5.5 ppm. Whether this glucose is itself linked to another glucose unit in position 6 to form a gentiobiose moiety or not is indicated by the chemical shift of protons in position 6, since protons at a glycosidation position are deshielded (Figure 5). Severe overlap of protons 2−5 of the esterified sugar unit prevents its positive identification by ^{1}H NMR. The anomeric proton of the second glucose unit of gentiobiose is found at *ca.* 4.2 ppm. The spin system of the second sugar unit could be simulated completely, and the large coupling constants between the ring protons places them in axial positions, which proves its identity as glucose. Table 6 reports the ^{1}H NMR data of the sugar protons. The exact substitution pattern of each crocetin derivative is

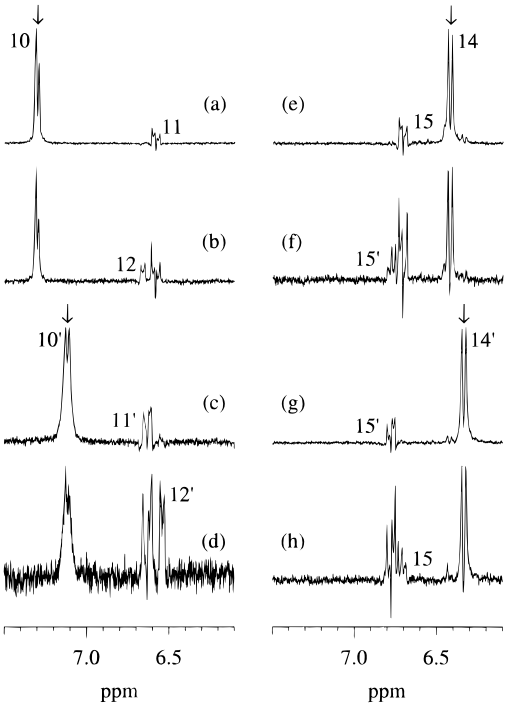

Figure 4. 500-MHz ^{1}H NMR 1D HOHAHA spectra of *all-trans*-crocetin mono(*β*-D-gentiobiosyl) ester **1d** in CD_3OD/C_6D_6 (4:1). The irradiated protons (arrow) are (a, b) 10, (c, d) 10′, (e, f) 14, and (g, h) 14′. Mixing times were (a, c, e, g) 9.4 ms and (b, d, f, h) 23.4 ms. A line broadening equal to half the digital resolution was applied by exponential multiplication.

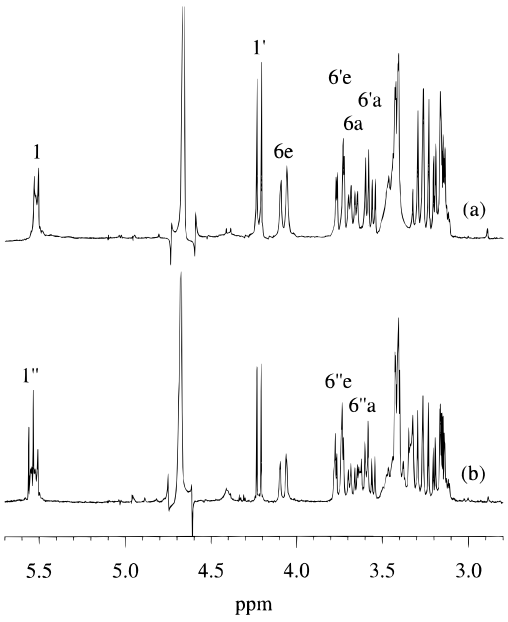

Figure 5. Sugar region of the resolution-enhanced ^{1}H NMR 1D spectra of crocetin derivatives in CD_3OD/C_6D_6 (4:1): (a) *all-trans*-crocetin di(*β*-D-gentiobiosyl) ester **1a**; (b) *all-trans*-crocetin *β*-D-gentiobiosyl-*β*-D-glucosyl ester **1b**.

given by integration of the two anomeric peaks with respect to that of the aglycon protons (Figure 6).

^{13}C NMR assignments followed from the HETCORR, HMQC, or CHORTLE and the COLOC or HMBC experiments. In the aglycon moiety long-range correlations were observed for the following pairs (and the corresponding ones on the other side of the molecule): C8/H1 (^3J), C8/H10 (^3J), C8/H19 (^3J), C9/H19 (^2J), C10/H11 (^2J), C10/H12 (^3J), C10/H19 (^3J), C11/H12 (^2J), C12/H10 (^3J), C12/H14 (^3J), C12/H20 (^3J), C13/H11 (^3J),

Table 5. ^{1}H NMR (300.13 MHz) Data for the Aglycon Moieties of Crocetin Esters in CD_3OD/C_6D_6 (4:1) at 25 °C[a]

proton	1a	1b	1c	1d	1e	2a	2b
10, 10′	7.352 br d[b] (11.6, 1.1, 2H)	7.356 br d (11.7, 0.4, 2H)	7.369 br d (11.8, 0.9, 2H)	7.370 br d (11.5, 1.1, 1H) 7.06[c] br d (11.9, 0.9, 1H)	7.333 br d (11.7, 1.6, 1H) 7.10[c] br d (12, 1, 1H)	7.424 br d (11.8, 1.5, 1H) 7.369 br d (11.6, 1.4, 1H)	7.417 br d (11.8, 1.2, 1H) 7.361 br d (11.6, 1.5, 1H)
11, 11′	6.474 dd (14.9, 11.6, 2H)	6.477 dd (14.9, 11.7, 2H)	6.480 dd (15.0, 11.8, 2H)	6.447 dd (15.1, 11.5, 1H) 6.522 dd (15.5, 11.9, 1H)	6.461 dd (14.9, 11.7, 1H) 6.518 dd (15, 12, 1H)	6.479 dd (15.0, 11.8, 1H) 6.466 dd (15.0, 11.6, 1H)	6.480 dd (14.7, 11.8, 1H) 6.466 dd (14.9, 11.6, 1H)
12, 12′	6.542 d (14.9, 2H)	6.540 d (14.9, 2H)	6.535 d (15.0, 2H)	6.540 d (15.1, 1H) 6.429 d (15.5, 1H)	6.537 d (14.9, 1H) 6.443 d (15, 1H)	7.124 d (15.0, 1H) 6.563 d (15.0, 1H)	7.125 d (14.7, 1H) 6.563 d (14.9, 1H)
14, 14′	6.285 br ∼dd (11.6, 1.0, −1.3, 2H)	6.282 br ∼dd (11.7, 1.2, −1.3, 2H)	6.274 br ∼dd (12.1, 0.9, −1.4, 2H)	6.269 br d (12, 1, −1, 1H) 6.171 br d (12, 1, −1, 1H)	6.274 br d (12, 1, −2, 1H) 6.214 br d (12, 1, −1, 1H)	6.142 br d (11.7, 0.8, 1H) 6.298 br d (11.4, 0.9, 1H)	6.143 br d (11.8, 0.8, 1H) 6.297 br d (11.8, 0.9, 1H)
15, 15′	6.626 ∼dd (14.1, 11.6, −1.3, 2H)	6.625 ∼dd (14.4, 11.7, −1.3, 2H)	6.622 ∼dd (14.7, 12.1, −1.4, 2H)	6.535 dd (15, 12, −1, 1H) 6.617 dd (15, 12, −1, 1H)	6.580 dd (14, 12, −2, 1H) 6.623 dd (14, 12, −1, 1H)	6.799 dd (14.2, 11.7, 1H) 6.526 dd (14.2, 11.4, 1H)	6.801 dd (13.8, 11.8, 1H) 6.526 dd (13.8, 11.8, 1H)
19, 19′	1.863 br s (1.1, 6H)	1.866 br s (0.4, 6H)	1.869 br s (0.9, 6H)	1.865 br s (1.1, 3H) 1.865 br s (0.9, 3H)	1.847 br s (1.6, 3H) 1.853 br s (1, 3H)	1.870 br s (1.5, 3H) 1.865 br s (1.4, 3H)	1.867 br s (1.2, 3H) 1.863 br s (1.5, 3H)
20, 20′	1.801 br s (1.0, 6H)	1.800 br s (1.2, 6H)	1.800 br s (0.9, 6H)	1.797 br s (1, 3H) 1.813 br s (1, 3H)	1.804 br s (1, 3H) 1.804 br s (1, 3H)	1.823 br s (0.8, 3H) 1.793 br s (0.9, 3H)	1.826 br s (0.8, 3H) 1.796 br s (0.9, 3H)

[a] δ multiplicity (J/Hz, number of protons). [b] Abbreviations: s, singlet; d, doublet; br, broad; ∼, first-order approximation. [c] The chemical shift of this proton varies from one sample preparation to another.

Table 6. ^{1}H NMR (300.13 MHz) Data for the Glucose Moieties of Crocetin Esters in CD_3OD/C_6D_6 (4:1) at 25 °C

proton	1a	1b	1c	1d	1e	2a	2b
1	5.52 ∼d[b] (8, 2H)	5.52 ∼d (8, 1H)		5.52 ∼d (8, 1H)		5.54 ∼d (8, 1H) 5.53 ∼d (8, 1H)	5.53[c] ∼d (8, 1H)
2, 3, 4, 5	3.4−3.5	3.4−3.5		3.4−3.5		3.4−3.5	3.4−3.5
6a	3.674 dd (−11.2, 4.3, 2H)	3.672 dd (−11.3, 4.4, 1H)		3.668 dd (−11.1, 5.0, 1H)		3.677 dd (−11.1, 3.3, 2H)	3.673 dd (−11.2, 3.8, 1H)
6e	4.077 dd (−11.2, 1.2, 2H)	4.077 dd (−11.3, 1.6, 1H)		4.101 dd (−11.1, 1.9, 1H)		4.081 dd (−11.1, 1.5, 2H)	4.074 dd (−11.2, 1.2, 1H)
1′	4.222 d (7.9, 2H)	4.220 d (8.1, 1H)		4.235 d (7.9, 1H)		4.223 d (8.0, 2H)	4.218 d (7.6, 1H)
2′	3.167 dd (9, 7.9, 2H)	3.167 dd (9, 8.1, 1H)		3.182 dd (9, 7.9, 1H)		3.184 dd (9, 8.0, 2H)	3.171 dd (9, 7.6, 1H)
3′	3.291 dd (9, 9, 2H)	3.289 dd (9, 9, 1H)		3.314 dd (9, 9, 1H)		3.298 dd (10, 9, 2H)	3.290 dd (9, 9, 1H)
4′	3.235 dd (9, 9, 2H)	3.234 dd (9, 9, 1H)		3.261 dd (9, 9, 1H)		3.242 dd (10, 9, 2H)	3.236 dd (9, 9, 1H)
5′	3.141 ddd (9, 5.7, 2.4, 2H)	3.139 ddd (9, 5.7, 2.5, 1H)		3.15		3.14 ddd (9, 5.9, 2.2, 1H)	3.136 ddd (9, 5.6, 2.3, 1H)
6′a	3.574 dd (−12.0, 5.7, 2H)	3.574 dd (−12.0, 5.7, 1H)		3.600 dd (−12.0, 5.5, 1H)		3.580 dd (−12.0, 5.9, 2H)	3.573 dd (−12.0, 5.6, 1H)
6′e	3.746 dd (−12.0, 2.4, 2H)	3.748 dd (−12.0, 2.5, 1H)		3.750 dd (−12.0, 2.5, 1H)		3.753 dd (−12.0, 2.2, 2H)	3.748 dd (−12.0, 2.3, 1H)
1″		5.55 ∼d (8, 1H)	5.55 ∼d (8, 2H)		5.50 ∼d (8, 1H)		5.52[c] ∼d (8, 1H)
2″, 3″, 4″, 5″		3.3−3.4	3.3−3.4		3.3−3.4		3.3−3.4
6″a		3.615 dd (−12.3, 6.4, 1H)	3.618 dd (−12.0, 4.7, 2H)		3.583 dd (−12.1, 4.8, 1H)		3.62 dd (−11, 4, 1H)
6″e		3.754 dd (−12.3, 1.8, 1H)	3.763 dd (−12.0, 1.5, 2H)		3.730 dd (−12.1, 1.4, 1H)		3.78 dd (−11, 2, 1H)

[a] δ multiplicity (J/Hz, number of protons). [b] Abbreviations: d, doublet; ∼, first-order approximation. [c] Assignments could be interchanged.

C13/H12 (^2J), C13/H20 (^2J), C14/H12 (^3J), C14/H15 (^2J) and/or C14/H15′ (^3J), C14/H20 (^3J), C15/H14 (^2J) and/or C15/H14′ (^3J), C15/H15′ (^2J), C19/H10 (^3J), C20/H12 (^3J), and C20/H14 (^3J). Tables 7 and 8 report ^{13}C chemical shifts of the aglycon and sugar carbons, respectively. The chemical shift values of the sugar carbons compare well with those of β-gentiobiose and β-glucose in the same solvent when esterification shifts are taken into account. These data provide evidence as to the identity of the carbohydrates esterifying the crocetin aglycons. However, a definitive proof that all sugar moieties are glucose is given by the coelution of the compounds purified in this study with enzymatically synthesized crocetin monoglucosyl **1e**, monogentiobiosyl **1d**, diglucosyl **1c**, gentiobiosylglucosyl **1b** and **2b**, and digentiobiosyl **1a** and **2a** esters, from UDP-glucose and crocetin, catalyzed by cell-free extract of saffron (Dufresne et al., 1997). ^{13}C chemical shifts are more

Table 7. ^{13}C NMR (75.47 MHz) Chemical Shifts (δ) of the Aglycon Carbons of Crocetin and Its Derivatives in DMSO-d_6 at 25 °C

carbon	multiplicity[a]	**1a**	**1b**	**1c**[b]	**1d**	**1f**	**2a**[b]
8, 8′	Q	166.26	166.19		166.26[c]	169.10	
9, 9′	Q	125.34	125.28, 125.34		124.87[c]	126.93	
10, 10′	T	140.00	139.91, 139.81	139.75	140.01[c]	138.02	140.09, 139.99
11, 11′	T	123.96	123.92	123.90	123.31[c]	124.09	125.11, 123.70
12, 12′	T	144.70	144.61, 144.54	144.49	144.81[c]	143.31	136.88, 144.77
13, 13′	Q	136.99	136.92		135.66, 138.28	136.64	
14, 14′	T	136.07	135.97	135.92	136.28, 132.62	135.28	134.61, 135.98
15, 15′	T	132.07	131.99	131.95	132.39, 130.08	131.61	130.79, 131.16
19, 19′	P	12.73	12.68	12.66	12.68[c]	12.77	12.72
20, 20′	P	12.59	12.55	12.54	12.48[c]	12.52	20.05, 12.54

[a] From DEPT experiments. Abbreviations: P, primary; S, secondary; T, tertiary; Q, quaternary. [b] No data available for quaternary carbons; sample quantity was only sufficient to run a DEPT. [c] Only carbons corresponding to the esterified end of the molecule are visible or chemical shifts are the same on both sides.

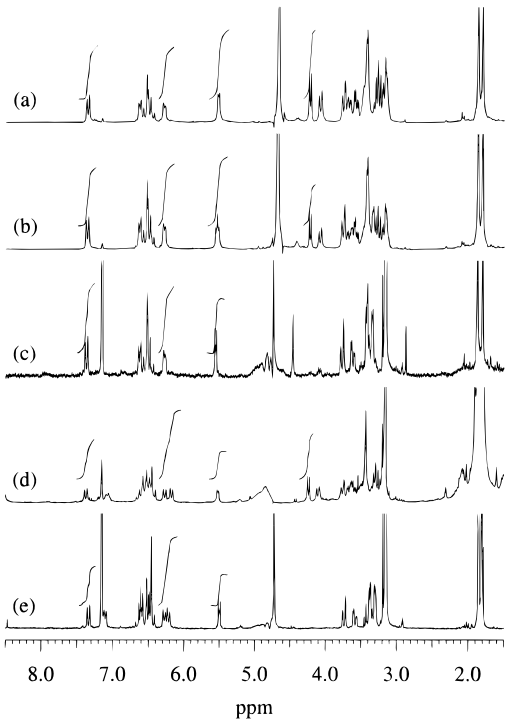

Figure 6. 1D ^{1}H NMR spectra of crocetin derivatives in CD_3OD/C_6D_6 (4:1) with integration: esters of *all-trans*-crocetin (a) di(β-D-gentiobiosyl) **1a**; (b) β-D-gentiobiosyl-β-D-glucosyl **1b**; (c) di(β-D-glucosyl) **1c**; (d) mono(β-D-gentiobiosyl) **1d**; (e) mono(β-D-glucosyl) **1e**. The spectra were obtained after exponential multiplication with a line broadening factor equal to the digital resolution.

sensitive than ^{1}H ones to different sugar substituents on each side of the aglycon. Some carbon peaks near the carbonyl end are doubled in the spectrum of compound **1b** (Table 7), and extra peaks that correspond to the glucose moiety are also present in the sugar region of the spectrum (Table 8). In compound **1d**, a few carbons (and their correlation to protons) on the free carboxylic acid side of the molecule are not visible, which means that their motion must be slowed down by an appreciable amount. Other carbons have chemical shifts quite different from those of compounds **1a** and **1f**, which could be viewed as models for each side of the molecule. The exact reason for this behavior remains unknown, but it occurs only in DMSO and not in the methanol−benzene mixture. Dimer formation or aggregation could be a possible explanation. Only a DEPT spectrum could be acquired with a reasonable signal-to-noise ratio for compounds **1c** and **2a**. The *cis* double bond in compound **2a** causes an upfield shift of 7.9 ppm for carbon 12 and a downfield shift of 7.5 ppm for carbon

Table 8. ^{13}C NMR (75.47 MHz) Chemical Shifts (δ) of the Sugar Carbons of Crocetin Derivatives in DMSO-d_6 at 25 °C

carbon	multiplicity[a]	**1a**	**1b**	**1c**	**1d**	**2a**
1	T	94.59	94.56		94.64	94.68
2	T	72.48	72.40		72.46	72.51
3	T	76.32	76.10		76.20	76.26
4	T	69.23	69.10		69.10	69.13
5	T	76.32	76.25		76.39	76.41
6	S	67.98	67.90		68.01	68.02
1′	T	103.14	103.06		103.18	103.21
2′	T	73.49	73.32		73.43	73.50
3′	T	76.75	76.63		76.67	76.74
4′	T	69.98	69.85		69.90	69.96
5′	T	76.90	76.83		76.96	76.98
6′	S	61.02	60.85		60.91	60.97
1″	T		94.56	94.54		
2″	T		72.40	72.39		
3″	T		76.25	76.26		
4″	T		69.40	69.39		
5″	T		77.80	77.79		
6″	S		60.43	60.41		

[a] From DEPT experiments. Abbreviations: P, primary; S, secondary; T, tertiary; Q, quaternary.

20, in agreement with previously reported values (Speranza et al., 1984). Unfortunately, there was not enough material for compounds **1e** and for the *cis* isomer **2b** to obtain useful ^{13}C NMR spectra.

CONCLUSION

Spectroscopic methods have proven useful to confirm the structure of crocetin derivatives from saffron stigmas and gardenia fruits. The combination of mass spectrometry giving the number of glucose moieties on the crocetin backbone, UV−vis spectroscopy detecting the presence of a *cis* double bond, and NMR spectroscopy with the use of one- and two-dimensional ^{1}H and ^{13}C experiments giving a complete molecular picture both qualitatively and quantitatively was sufficient to identify seven of the compounds that could be isolated.

In compound **2b**, whether the *cis* double bond is on the gentiobiose or the glucose side of the molecule is unclear, the 19−10−11−12 or 19′−10′−11′−12′ and 20−14−15−15′−14′−20′ spin systems not being connected. The presence of both isomers simultaneously cannot be ruled out, but only one *cis* double bond is present. All attempts to observe nuclear Overhauser enhancements using NOE difference or ROESY experiments between the anomeric proton of the esterified glucose moieties and one of the aglycon protons have been unsuccessful.

ACKNOWLEDGMENT

We are grateful to Takashige Shimizu, President of San-Ei Gen F.F.I., Inc. We thank Christopher A. Barr for acquiring the crocetin NMR spectra and Dr. Irena

H. Ekiel of the Biotechnology Research Institute of the National Research Council of Canada in Montreal, PQ, Canada, for time on the 500-MHz NMR spectrometer.

LITERATURE CITED

Aue, W. P.; Bartholdi, E.; Ernst, R. R. Two-dimensional spectroscopy. Application to nuclear magnetic resonance. *J. Chem. Phys.* **1976**, *64*, 2229−2246.

Bax, A.; Freeman, R. Investigation of complex networks of spin-spin coupling by two-dimensional NMR. *J. Magn. Reson.* **1981**, *44*, 542−561.

Bax, A.; Morris, G. A. An improved method for heteronuclear chemical shift correlation by two-dimensional NMR. *J. Magn. Reson.* **1981**, *42*, 501−505.

Bax, A.; Summers, M. F. ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094.

Bax, A.; Griffey, R. H.; Hawkins, B. L. Correlation of proton and nitrogen-15 chemical shifts by multiple quantum NMR. *J. Magn. Reson.* **1983**, *55*, 301−315.

Bendall, M. R.; Pegg, T. D.; Doddrell, D. M. Pulse sequences utilizing the correlated motion of coupled heteronuclei in the transverse plane of the doubly rotating frame. *J. Magn. Reson.* **1983**, *52*, 81−117.

Connors, R. E.; Burns, D. S.; Farboosh, R.; Frank, H. A. Computational studies of the molecular structure and electronic spectroscopy of carotenoids. *J. Phys. Chem.* **1993**, *97*, 9351−9355.

Davis, D. G.; Bax, A. Simplification of ^{1}H NMR spectra by selective excitation of experimental subspectra. *J. Am. Chem. Soc.* **1985**, *107*, 7198−7199

Doddrell, D. M.; Pegg, D. T.; Bendall, M. R. Distortionless enhancement of NMR signals by polarization transfer. *J. Magn. Reson.* **1982**, *48*, 323−327.

Dufresne, C.; Cormier, F.; Dorion, S. *In vitro* formation of crocetin glucosyl esters by *Crocus sativus* L. callus extract. *Planta Med.* **1997**, in press.

Ichi, T.; Higashimura, Y.; Koda, T. Food chemical properties of gardenia yellow pigments. *Foods Food Ingredients J. Jpn.* **1995**, 96−103.

Kessler, H.; Griesinger, C.; Zarbock, J.; Loosli, H. R. Assignment of carbonyl carbons and sequence analysis in peptides by heteronuclear shift correlation via small coupling constants with broadband decoupling in t_1 (COLOC). *J. Magn. Reson.* **1984**, *57*, 331−336.

Pearson, G. A. High-accuracy proton-carbon chemical-shift correlations from one-dimensional polarization-transfer ^{13}C NMR spectra. *J. Magn. Reson.* **1985**, *64*, 487−500.

Pfander, H.; Rychener, M. Separation of crocetin glycosyl esters by high-performance liquid chromatography. *J. Chromatogr.* **1982**, *234*, 443−447.

Pfander, H.; Wittwer, F. Carotinoid-Glycoside: Untersuchungen zur Carotinoid-Zusammensetzung im Safran. *Helv. Chim. Acta* **1975a**, *58*, 1608−1620.

Pfander, H.; Wittwer, F. Carotinoid-Glycoside: Untersuchungen zur Carotinoidzusammensetzung im Safran. *Helv. Chim. Acta* **1975b**, *58*, 2233−2236.

Pfister, S.; Meyer, P.; Steck, A.; Pfander, H. Isolation and structure elucidation of carotenoid-glycosyl esters in gardenia fruits (*Gardenia jasminoides* Ellis) and saffron (*Crocus sativus* Linne). *J. Agric. Food Chem.* **1996**, *44*, 2612−2615.

Shaka, A. J.; Keeler, J.; Freeman, R. Evaluation of a new broadband decoupling sequence: WALTZ-16. *J. Magn. Reson.* **1983**, *53*, 313−340.

Shaka, A. J.; Barker, P. B.; Freeman, R. Computer-optimized decoupling scheme for wideband applications and low-level operation. *J. Magn. Reson.* **1985**, *64*, 547−552.

Speranza, G.; Dadà, G.; Manitto, P.; Monti, D.; Gramatica, P. 13-*cis*-crocin: a new crocinoid of saffron. *Gazz. Chim. Ital.* **1984**, *114*, 189−192.

Tarantilis, P. A.; Tsoupras, G.; Polissiou, M. Determination of saffron (*Crocus sativus* L.) components in crude plant extract using high-performance liquid chromatography-UV-visible photodiode-array detection-mass spectrometry. *J. Chromatogr. A* **1995**, *699*, 107−118.

Timberlake, C. F.; Henry, B. S. Plant pigments as natural food colors. *Endeavour* **1986**, *10*, 31−36.

Received for review May 9, 1996. Revised manuscript received December 30, 1996. Accepted December 30, 1996.[⊗] This study was supported in part by a grant from the Canadian Ministry of External Affairs and International Trade within the scope of a research collaboration with San-Ei Gen F.F.I., Inc. of Osaka, Japan.

JF9603487

[⊗] Abstract published in *Advance ACS Abstracts*, March 1, 1997.