ELSEVIER

Dyes and Pigments 49 (2001) 15–20

DYES and PIGMENTS

www.elsevier.com/locate/dyepig

Isolation and characterization of the major colorant in Gardenia fruit

Heung-Jin Choi [a,*], Yeon Sil Park [a], Moon Goo Kim [a],
Tae Kyung Kim [b], Nam Sik Yoon [b], Yong Jin Lim [b]

[a] *Department of Industrial Chemistry, Kyungpook National University, Taegu 702-701, South Korea*
[b] *Department of Dyeing and Finishing, Kyungpook National University, Taegu 702-701, South Korea*

Received 14 April 2000; received in revised form 9 January 2001; accepted 26 January 2001

Abstract

The major yellow colorant from the fruit of *Gardenia jasminoides* was isolated and then identified with the aid of FAB–MS, UV/visible and NMR data. The NMR assignments were based on data from ^{1}H NMR, ^{13}C NMR, DQF–COSY, NOESY, HMQC and HMBC measurements. Molar absorptivity was determined using an analytically pure sample of crocin. © 2001 Published by Elsevier Science Ltd.

Keywords: Crocin; *Gardenia jasminoides*; Molar absorptivity; Structure determination

1. Introduction

Yellow colorants from *Gardenia jasminoides* that are suitable for foodstuffs and natural dye preparations have been postulated as crocetin glycosyl esters, using data from HPLC and absorption studies [1]. Although the main colorant from saffron was identified as the digentiobiosyl ester of crocetin [2], the detailed structure of yellow pigments from Gardenia fruit was only reported recently.

In 1995, the structures of crocetin derivatives from Gardenia fruit was characterized using LC/MS and ^{1}H NMR data [3]. Following their cleavage from the attached colorants, the sugar moieties were analyzed chromatographically by comparisons with authentic sugar samples. Pfander and coworkers reported the structure of peracetylated crocin based on UV/visible, ^{1}H and ^{13}C NMR, and FAB–MS data [4].

In the present paper, we describe the isolation and structure determination of crocin itself, beginning with the fruit of fully matured *Gardenia jasminoides*. The utility of carotenoid–glycosyl esters as colorants for textiles [5] and food [6,7], as antioxidant quenchers for free radicals [8], and as an antitumor agent [9] makes the identification of crocin of considerable interest.

2. Experimental

2.1. General

Medium pressure preparative liquid chromatography (MPLC) was performed with a Yamazen YFLC-540 system equipped with a C-18 silica gel

---

* Corresponding author. Tel.: +82-53-950-5582; fax: +82-53-950-6594.
E-mail address: choihj@knu.ac.kr (H.-J. Choi).

PII: S0143-7208(01)00007-9

column (20×300 mm; Yamazen ODS-S-50B) and a fraction collector. High-performance liquid chromatography (HPLC) was performed with a Jasco low-pressure gradient system, a PU-980 pump, and a UV-975 UV/vis detector. Separation and purification of crocin were conducted via semi-preparative HPLC using a Jasco gradient system with a C-18 column (Supelco LC-18-DB, 25×100 mm, 5 μm, 120 Å). UV/vis spectra were recorded on a Shimadzu UV 2100 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz) and Varian Unity Inova 500 spectrometer (500 MHz for ^{1}H NMR). The 2D NMR experiments were performed on a Bruker Avance 400 spectrometer (400 MHz) equipped with a 5 mm QNP probe using standard Bruker pulse programs. Chemical shifts are reported as δ values with reference to tetramethylsilane (TMS) as internal standard. ^{1}H–^{1}H shift correlation 2D DQF–COSY spectra were obtained using the spectral widths of 3592 Hz. For each t1 increment, 16 scans were accumulated and initial (t1, t2) matrices of 256×1024 real data points were zero-filling to 1024×1024. ^{1}H–^{1}H NOESY experiments (mixing time of 750 ms) were recorded in the phase sensitive mode with time proportional phase incrementation (TPPI). The acquisition and processing parameters were the same as in the DQF–COSY experiment. ^{1}H–^{1}H homonuclear 2D *J*-resolved (HH2DJR) spectra were obtained using the F2 spectral width of 40 Hz. One bond ^{1}H–^{13}C HMQC spectra were obtained using B_0 gradient pulse for the selection of ^{1}H coupled to ^{13}C carbons. For each t1 increment, 16 scans were accumulated with resolution by collecting 516 data sets. The F1 and F2 spectral widths were 17602 and 3397 Hz, respectively. Heteronuclear multiple bond correlation HMBC spectra were recorded using a delay for the long range coupling (mixing time of 50 ms). As in the HMQC experiment, a B_0 gradient pulse was applied. Except for the sequence and the delays mentioned, all parameters were the same as in the HMQC experiment.

2.2. Extraction and isolation of crocin from Gardenia

Ground dried Gardenia fruit (10 g) was extracted successively with methylene chloride (150 ml) and methanol (200 ml), and the methanol extract was evaporated to dryness to give a reddish yellow viscous oil (2.20 g), which was dissolved in water and filtered through a layer of celite. A portion (0.5 ml) of the reddish yellow filtrate (20 ml) was applied to the semi-preparative C-18 column. The column was eluted with a mixture of water–methanol (1:1) for 15 min and then for 50 min with a linear gradient of methanol–water (1:1) to 100% methanol (HPLC grade) at 2.5 ml/min. The HPLC chromatogram was monitored at 433 nm and showed peaks having retention times and percent compositions of 16.1 (60.5%), 21.3 (1.0), 23.9 (13.6), 28.2 (0.7), 35.2 (20.0), 37.1 (2.0), 41.5 (1.6), and 42.5 (0.6). The major yellow component (retention time = 16.1 min) was concentrated to dryness under high vacuum at room temperature to give analytically pure yellow solid crocin (7 mg). When a larger scale isolation of crocin was performed using MPLC, a 10-ml fraction of the yellow extract gave 51 mg of pure crocin.

2.3. Mass spectrometry

The fast atom bombardment (FAB) mass spectrum of crocin was obtained on a Jeol JMS-700 double-focussing high-resolution mass spectrometer using a xenon gun (6 K eV, 12 mA emission current, 50°C chamber temperature) and glycerol matrix. HR FAB MS ($[M+H]^{+}$): calc. = 977.387; found = 977.385.

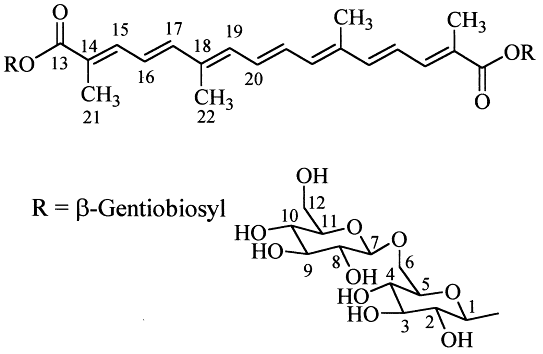

Fig. 1. The structure of crocin (crocetin di-(β-gentiobiosyl) ester).

2.4. UV/visible and elemental analysis

Elemental analysis on pure crocin was performed by Desert Microanalytics Laboratory (Tucson, Arizona, USA), and the absorption spectrum was recorded in methanol (HPLC grade) using a concentration of 4.985×10^{-6} mol/l. $\lambda_{\max}$ ($\varepsilon_{\max}$) = 458 nm (96,640), 433 nm (103,480). Anal. calc. for $C_{44}H_{64}O_{24}\cdot 2H_2O$: C, 52.17; H, 6.77. Found: C, 52.02; H, 6.47.

2.5. Structure determination of crocin

The structure of crocin was further established by NMR. The NMR sample was prepared by dissolving pure crocin (3.5 mg) in deaerated DMSO-d_6 (99.9%, 0.5 ml). The ^{1}H NMR spectrum of crocin in DMSO-d_6–D_2O (5:3) was also recorded on a Varian Unity Inova 500 MHz spectrometer.

3. Results and discussion

Although eight yellow crocetin derivatives were detected by HPLC analysis of an extract of fully matured *Gardenia jasminoides*, only three of them were present in significant quantities. Previously, Ichi et al. had reported that seven yellow components from fully matured Gardenia fruit were crocetin esters. The three main components were identified as crocetin digentiobioside ester (crocin), crocetin monogentiobioside monoglucoside ester and crocetin diglucoside ester [3]. In our studies, the retention times (RT) and proportions for these

Table 1
NMR data for the β-digentibiosyl component of crocin

Position	^{13}C (DMSO)	^{1}H (500 MHz, DMSO/D_2O)	^{1}H (400 MHz, DMSO)	HMBC	NOESY
1	94.9	5.47 *d* (8.2)	5.42 *d* (7.6)	C-2, C-5, C-13	H-3, H-5
2	72.8	3.36 *m*	3.20 *m* OH 5.31 d (4.8)	C-3	H-4
3	76.61	3.38 *m*	3.22 *m* OH 5.08 d (5.2)	C-5	H-1, H-5
4	69.5	3.40 *m*	3.24 *m* OH 5.17 d (4.4)	C-3, C-5	H-2, H-6, H-6′
5	76.58	3.54 *m*	3.43 *m*	–	H-1, H-3
6	68.2	3.70 *dd* (11.0, 5.0) 4.04 *br dd* (11.0, 1.8)	3.59 *dd* (11.2, 5.2) 3.99 *br d* (10.0)	C-5, C-7	H-4, H-7
7	103.4	4.28 *d* (8.0)	4.17 *d* (8.0)	C-6, C-8, C-11	H-6, H-6′, H-9, H-11
8	73.8	3.09 *dd* (8.0, 4.8)	2.94 *ddd* (9.3, 8.0, 4.8) OH 4.85 *d* (4.8)	C-7, C-9	H-10
9	77.1	3.27 *dd* (9.2, 8.8)	3.11 *m* OH 4.92 *d* (4.4)	C-10, C-11	H-7, H-11
10	70.3	3.15 *dd* (9.8, 8.8)	3.05 *m* OH 4.88 *d* (4.4)	C-9, C-11	H-8, H-12
11	77.2	3.21 *ddd* (9.8, 6.0, 1.9)	3.05 *m*	C-9, C-10	H-7, H-9
12	61.3	3.52 *dd*, (6.0, 12.8) 3.74 *br dd* (12.8, 1.9)	3.42 *o dd* 3.65 *ddd* (11.2, 6.0, 2.0) OH 4.44 *t* (6.0)	–	H-10
13	166.5	–	–	H-1, H-15, H-21	–
14	125.6	–	–	H-21	–
15	140.3	7.42 *d* (11.6)	7.36 *d* (11.6)	C-13, C-17, C-21	H-17, H-19
16	124.3	6.69 *dd* (11.6, 14.5)	6.67 *dd* (11.6, 14.5)	C-17, C-18	H-21, H-22
17	145.0	6.85 *d* (14.5)	6.82 *d* (14.5)	C-15, C-16, C-18, C-19, C-22	H-15, H-19
18	137.3	–	–	H-16, H-17, H-22	–
19	136.3	6.58 *m* (AA′XX′)	6.53 *m* (AA′XX′)	C-17, C-20, C-22	H-15, H-17
20	132.4	6.88 *m* (AA′XX′)	6.88 *m* (AA′XX′)	C-19	H-22
21	13.0	1.98 *s*	1.97 *s*	C-13, C-14, C-15	H-16
22	12.9	2.00 *s*	2.00 s	C-17, C-18, C-19	H-16, H-20

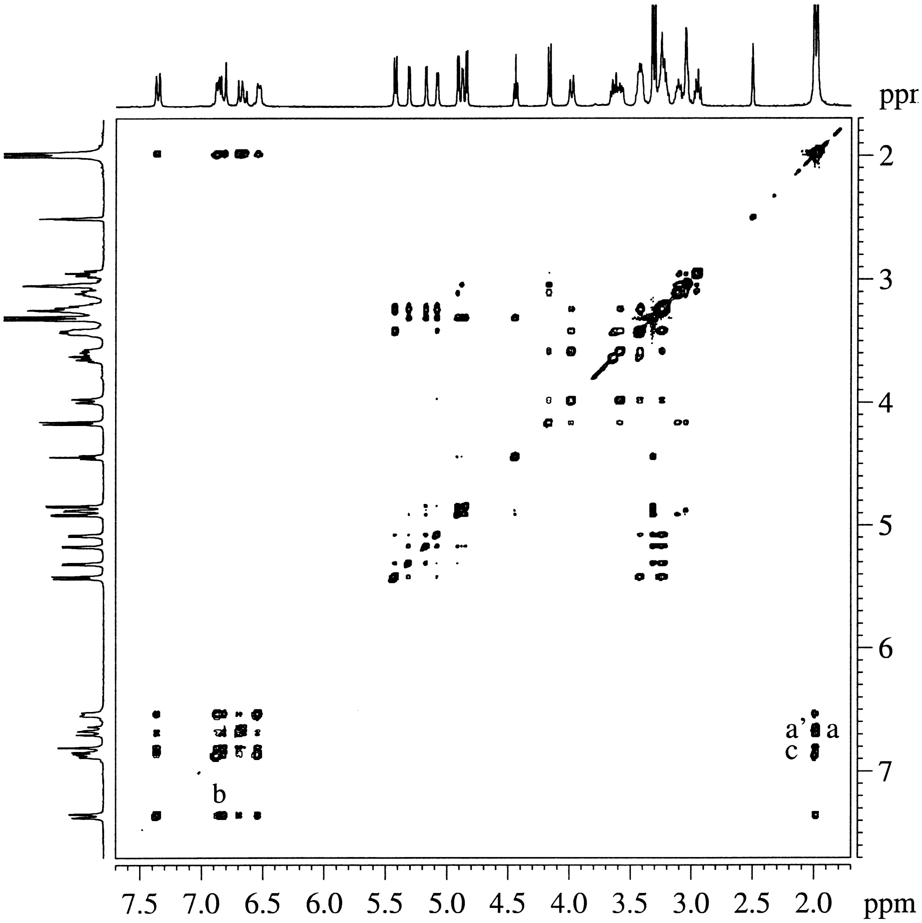

Fig. 2. ^1H–^1H NOESY spectrum of crocin in DMSO-d_6.

components were crocin (RT 16.1, 60.5%), crocetin monogentiobioside monoglucoside ester (RT 23.9, 13.6%), and crocetin diglucoside ester (RT 35.2, 20.0%). Our assignments were based on HPLC data, as the hydrophilic character of each component is appreciably different. The crocetin component having the greater level of carbohydrate character had the lowest RT on C-18.

The major component, crocin, was isolated as a reddish yellow amorphous powder. The HR–FAB MS afforded a molecular ion ($[M-H]^+$) peak at 977.387 and the results of microanalysis were consistent with the molecular formula $C_{44}H_{64}O_{24}$. The UV–visible spectrum of crocin in methanol exhibited absorption bands at 458 nm and 433 nm with a shoulder at 410 nm, which is consistent with results of other studies [3,4,6]. A molar absorptivity of 103,480 has been reported for the absorption at 433 nm.[1]

Crocin has a two-fold axis of symmetry (C_2), making it a chiral molecule (Fig. 1). The ^1H and ^{13}C NMR spectra (Table 1) indicated the presence of a disaccharide moiety (δ_H 5.47, 4.28–3.09; δ_C 61.3–103.4), a conjugated polyene moiety (δ_H 6.58, 6.69, 6.85, 6.88, 7.42; δ_C, 124.3, 125.6, 132.4, 136.3, 137.3, 140.3, 145.0), two types of methyl groups (δ_H 1.97, 2.00), and a conjugated carbonyl carbon (δ_C

---

[1] In Ref. [3] the relative absorptivity coefficient of crocin is reported as $E_{1\%}^{1\mathrm{cm}}$ 4320, which is the absorptivity representing the absorbance of a 1% solution in a 1 cm path-length cell, and corresponding to molar absorptivity, ε 14,200.

166.5). ^{1}H NMR did not give completely resolved resonance signals, especially for the carbohydrate groups. However, from HH2DJR data, the proton chemical shifts and associated coupling constants were determined and all chemical shifts could be assigned. Since some proton signals overlapped, 2D NMR spectra were employed to assign the proton and carbon chemical shifts unambiguously. ^{1}H–^{1}H DQF–COSY, NOESY, HH2DJR, HMQC and HMBC techniques were used, and the resultant ^{1}H and ^{13}C chemical shifts are listed in Table 1.

The ^{1}H NMR spectrum of carefully dried crocin in deuterated DMSO solution shows well-resolved signals arising from hydroxyl protons (δ_{OH} 4.44, 4.85, 4.88, 4.92, 5.08, 5.17 and 5.31), all of which disappeared following the addition of deuterium oxide. Only the hydroxyl group at 4.44 ppm was observed as a triplet, indicating that a hydroxymethyl group was present. This assignment was unambiguously confirmed using NOESY (Fig. 2) and proton–carbon long-range coupling (HMBC) experiments.

The proton chemical shifts reported in Table 1 are taken from the 500 MHz spectrum, even though ^{13}C and 2D NMR spectra were recorded at 400 MHz. Anomeric proton signals at 5.42 (C-1) and 4.17 (C-7) ppm were clearly distinguished and the results of integration suggest that the saccharide is a glycoside. The anomeric protons are coupled (7.6 Hz for δ 5.42 and 8.0 Hz for δ 4.17)

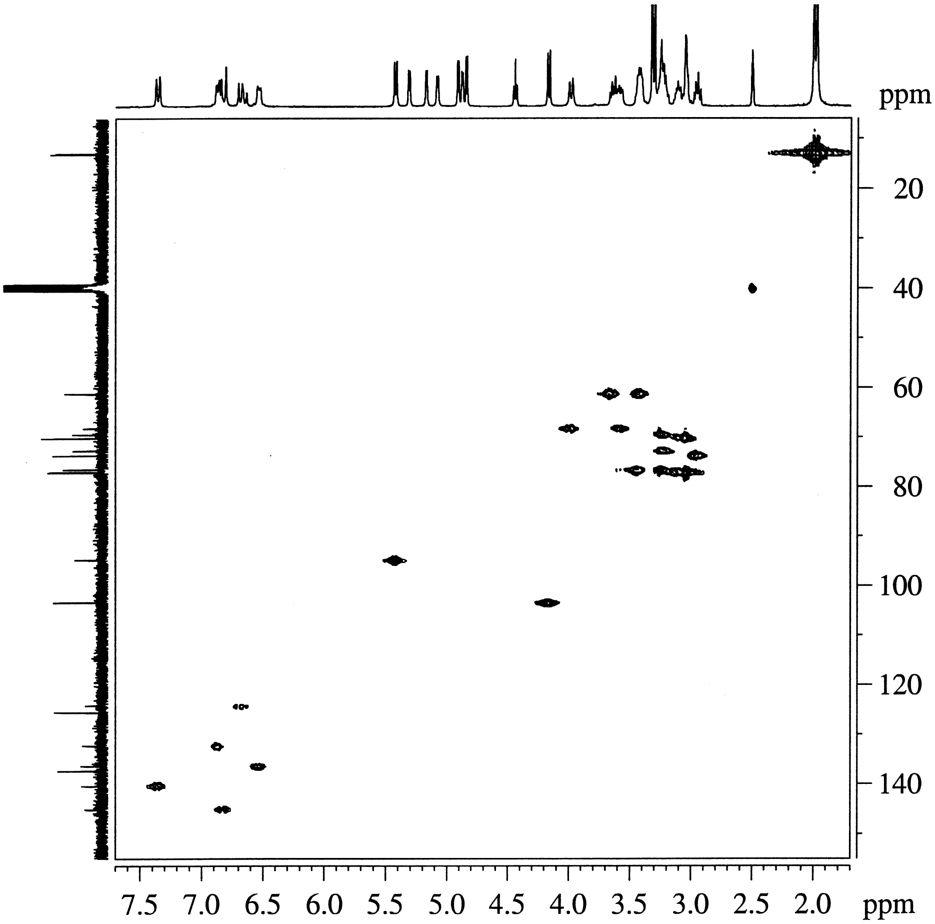

Fig. 3. Gradient selected ^{1}H–^{13}C HMQC spectrum of crocin in DMSO-d_6.

with neighboring vicinal protons (C-2 and C-8), suggesting that the configuration at C-1 and C-7 corresponds to that of a β-glycoside.

The chemical shift assignments in the carbohydrate moiety were achieved with the aid of DQF–COSY and HMQC spectra (Fig. 3), and showed that two distinct hexoses were present. Carbon and proton (C;H) signals for one of them were 94.9; 5.42, 72.8; 3.20, 76.61; 3.22, 69.5; 3.24, 76.58; 3.43, 68.2; 3.59 and 3.99, and the (C;H) signals for the second were 103.4; 4.17, 73.8; 2.94, 77.1;3.11, 70.3; 3.05, 77.2; 3.05, 61.3; 3.42 and 3.65. The coupling constants for all vicinal protons within the each sugar moieties were $\geqslant 7.0$ Hz. These values indicate that the protons in the pyran rings occupy axial positions. Each hydroxyl group was readily assigned by DQF–COSY and HMQC spectra.

In NMR experiments involving the carotenoid moiety of crocin, distinct ABC and AA′BB′ spin systems were observed from the coupling patterns, and two types of methyl groups were present. From ^{1}H, ^{13}C NMR and HMQC spectra, the following carbon to proton (C;H) correlations were observed: 13.0;1.97 and 12.9;2.00 (methyl groups), 140.3;7.36, 124.3;6.67 and 145.0;6.82 (ABC spin system), 136.3;6.53 and 132.4;6.88 (AA′BB′ spin system), as well as 166.5 (carbonyl), 125.6 and 137.3 (quaternary carbons). From DQF–COSY and HMQC spectra, the skeletal connection could be established and the configuration of the carotenoid moiety was determined by NOESY experiment. The vinyl proton on C-16 at 6.67 ppm showed NOE enhancement by the methyl protons (C-21 and C-22) at 1.97 and 2.00 ppm (see a and a′ in Fig. 2), and the vinyl protons on C-15 (δ 7.36) and C-17 (δ 6.82) showed NOE enhancement with respect to each other (see b in Fig. 2) but no NOE involving the methyl groups. NOE interaction between the C-20 proton (δ 6.88) and methyl protons on C-22 (δ 2.00), labeled as c in Fig. 2, was also observed. These observations indicate that crocetin has the *Z* configuration. Further evidence for this conclusion was obtained from the coupling constants (11.6 and 14.5 Hz) of the vinyl protons. The observed vicinal couplings for vinyl protons were greater than 11 Hz, indicating that the 1,2-disubstituted double bond is all *trans*.

4. Conclusions

While the results of NMR data generated in this investigation were consistent with data pertaining to the chemical structure of the crocin isolated from *Gardenia jasminoides* Ellis, the complete chemical assignment of crocin itself was reported for the first time. In addition, the molar absorptivity of analytically pure crocin has been measured. With the aid of this $\varepsilon_{\max}$ value, the crocetin derivatives in Gardenia saffron can be more correctly and easily quantified using analytical techniques such as LC/MS.

Acknowledgements

The authors acknowledge the financial support of the Ministry of Commerce, Industry and Energy provided in the program year 1998.

References

[1] (a) Nishizawa M, Chonan T, Sekijo I, Sugii T. Hokkaidoritsu Eisei Kenkyushoho 1983;33:28. (b) Kamikura M, Nakazato K. Eisei Shikensho Hokoku 1985;103:157.

[2] (a) Karrer P, Miki K. Helv Chim Acta 1929;12:985. (b) Kuhn R, L'Orsa F. Ber Deutsche Chem Ges 1931;64:1732.

[3] (a) Ichi T, Higashimura Y, Katayama T, Koda T, Shimizu T, Tada M. Nippon Shokuhin Kagaku Kogaku Kaishi 1995;42:776. (b) Ibid. 1995;42:784.

[4] Pfister S, Meyer P, Steck A, Pfander H. J Agric Food Chem 1996;44:2612.

[5] Liakopoulou-Kyriakides M, Tsatsaroni E, Laderos P, Georgiadou K. Dyes and Pigments 1998;36:215.

[6] Ichi T, Higashimura Y, Katayama T, Koda T, Shimizu T, Tada M. Nippon Shokuhin Kagaku Kogaku Kaishi 1995;42:784.

[7] Dufresne C, Cormier F, Dorion S, Niggli UA, Pfister S, Pfander H. Enzyme Microb Technol 1999;24:453.

[8] (a) Tubaro F, Micossi E, Ursini F. J Am Oil Chem. Soc 1996;73:173. (b) Pham TQ, Cormier F, Farnworth E, Tong VH, Calsteren MV. J Agric Food Chem 2000;48:1455.

[9] Escribano J, Alonso GL, Miguel CP, Fernandez JA. Cancer Lett 1996;100:23.