Novel Glycosidic Constituents from Saffron

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The methanolic extract of saffron was prefractionated with the aid of multilayer coil countercurrent chromatography (MLCCC). After subsequent purification of certain fractions by high-performance liquid chromatography, the following glycoconjugates were isolated and identified on the basis of their spectral (UV, MS, NMR) data: the β-D-glucosides of (4*R*)-4-hydroxy-3,5,5-trimethylcyclohex-2-enone (**5**), (4*S*)-4-hydroxy-3,5,5-trimethylcyclohex-2-enone **(6)**, and (4*S*)-4-(hydroxymethyl)-3,5,5-trimethylcyclohex-2-enone (**7**), as well as the β-D-gentiobiosyl ester of 2-methyl-6-oxohepta-2,4-dienoic acid (**4**). In addition to these new natural products, known saffron constituents, i.e., the di-β-D-gentiobiosyl and β-D-gentiobiosyl-β-D-glucopyranosyl esters of crocetin, have also been isolated and separated using MLCCC.

Keywords: *(4R)-4-Hydroxy-3,5,5-trimethylcyclohex-2-enone 4-O-β-D-glucopyranoside; (4S)-4-hydroxy-3,5,5-trimethylcyclohex-2-enone 4-O-β-D-glucopyranoside; (4S)-4-(hydroxymethyl)-3,5,5-trimethylcyclohex-2-enone 4-O-β-D-glucopyranoside; β-D-gentiobiosyl ester of 2-methyl-6-oxohepta-2,4-dienoic acid; crocetin derivatives; carotenoid degradation products; multilayer coil countercurrent chromatography; saffron; Crocus sativus; Iridaceae*

INTRODUCTION

The chemical composition of saffron, the dried stigmata of *Crocus sativus*, has been thoroughly studied by several groups, and considerable knowledge about saffron constituents (Zarghami and Heinz, 1971; Buchecker and Eugster, 1973; Pfander and Wittwer, 1975a,b and references cited; Rödel and Petrzika, 1991), their analysis (Pfander and Rychener, 1982; Oberdieck, 1991; Sujata et al., 1992; Castellar et al., 1993; Tarantilis et al., 1994; Alonso et al., 1996), stability (Tsimidou and Tsatsaroni, 1993; Morimoto et al., 1994), and biogenesis (Pfander and Schurtenberger, 1982) has been accumulated. The renewed interest in saffron presently is mainly contributed to its distinct anticancer activity (Morjani et al., 1990; Nair et al., 1992; Tarantilis et al., 1992).

Saffron is traditionally used for coloring and flavoring purposes, with mono- and diglycosyl esters of crocetin (2,6,11,15-tetramethylhexadeca-2,4,6,8,10,12,14-heptaenedioic acid, **1**) being the principal coloring components. Responsible for the typical flavor of saffron are the bitter tasting glucoside picrocrocin, i.e., the β-D-glucopyranoside of hydroxy-β-cyclocitral, and its degradation product, the odor-active safranal. For the formation of these carotenoid degradation products an oxidative cleavage of zeaxanthin has been proposed (Pfander and Schurtenberger, 1982) assuming the action of a hypothetic 7,8-carotenase. In the course of our ongoing studies on carotenoid degradation, saffron was chosen as substrate to investigate the regioselectivity of the bio-oxidative cleavage of saffron carotenoids. As a first result, the identification of four novel glycoconjugated carotenoid breakdown products in the stigmata of *Crocus sativus* is reported.

EXPERIMENTAL PROCEDURES

Materials. Dried saffron, type *electus pulvis* was purchased from a local supplier and stored in the dark at −18 °C. All commercial chemicals used were of analytical grade quality. Solvents were redistilled before use.

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Extraction of Saffron and Preseparation Using Multilayer Coil Countercurrent Chromatography (MLCCC). Saffron (8.8 g) was successively extracted with petroleum ether (250 mL), diethyl ether (250 mL), and methanol (3 × 250 mL) in the absence of light using a Soxhlet extractor. The methanolic extract was concentrated under reduced pressure to dryness and fractionated by preparative MLCCC (75 m × 2.6 mm i.d. PTFE tubing) using $CHCl_3$/MeOH/H_2O (7:13:8) as the solvent system (Ito, 1986); for details, cf. Roscher and Winterhalter (1993). Seventy 10 mL fractions have been collected which were combined as follows: 1−12 (fraction I), 13−29 (fraction II), 30−34 (fraction III), 35−64 (fraction IV), and 65−70 (fraction V).

Isolation of Crocetin Glycosyl Esters. Acetylation (Ac_2O in pyridine, room temperature) of MLCCC fraction I followed by flash chromatography and purification by HPLC on an Eurospher Si 100 column (5 μm, 250 × 16 mm, Knauer, Berlin, flow rate 10 mL/min, eluent: hexane/MTBE-gradient, UV detection 430 nm) yielded the peracetylated digentiobiosyl ester of crocetin **2** (15.5 mg).

Spectral data of peracetylated **2**: ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.99 (6 H, d, J = 1.0 Hz, CH_3-C2/C15), 2.01 (6 H, s, CH_3-C6/C11), 2.02−2.08 (42 H, 14 acetates), 3.60 (2 H, dd, J = 11.5, 5.5 Hz, H_aC6′), 3.64 (2 H, ddd, J = 9.5, 5.0, 2.5 Hz, HC5″), 3.84 (2 H, ddd, J = 10.0, 5.5, 2.5 Hz, HC5′), 3.94 (2 H, dd, J = 11.5, 2.5 Hz, H_bC6′), 4.10 (2 H, dd, J = 12.5, 2.5 Hz, H_aC6″), 4.25 (2 H, dd, J = 12.5, 5.0 Hz, H_bC6″), 4.56 (2 H, d, J = 8.0 Hz, HC1″), 4.99 (2 H, dd, J = 9.5, 8.0 Hz, HC2″), 5.02 (2 H, dd, J = 10.0, 9.5 Hz, HC4′), 5.06 (2 H, dd, J = 9.5, 9.5 Hz, HC4″), 5.19 (2 H, dd, J = 9.5, 9.5 Hz, HC3″), 5.21 (2 H, dd, J = 9.5, 8.0 Hz, HC2′), 5.28 (2 H, dd, J = 9.5, 9.5 Hz, HC3′), 5.75 (2 H, d, J = 8.0 Hz, HC1′), 6.43 (2 H, dd, J = 10.0, 2.0 Hz, HC7/HC10), 6.55 (2 H, dd, J = 15.0, 11.5 Hz, HC4/HC13), 6.69 (2 H, d, J = 15.0 Hz, HC5/HC12), 6.74 (2 H, m, HC8/HC9), 7.34 (2 H, dq, J = 11.5, 1.0 Hz, HC3/HC14); ^{13}C NMR (63 MHz, $CDCl_3$) δ 12.6 (CH_3-C2/C15), 12.8 (CH_3-C6/C11), 61.8 (C6″), 67.6 (C6′), 68.4 (C4″), 68.7 (C4′), 70.2 (C2′), 70.9 (C2″), 71.9 (C5″), 72.8 (C3′/C3″), 74.0 (C5′), 92.0 (C1′), 100.6 (C1″), 123.5 (C4/C13), 124.5 (C2/C15), 131.8 (C8/C9), 136.3 (C7/C10), 136.9 (C6/C11), 141.4 (C3/C14), 145.3 (C5/C12), 166.0 (C1/C16), 20.6−20.7 and 169.3−170.6 (14 acetates). Assignments were confirmed by C−H COSY and HMBC, thus indicating that previously published chemical shift data for C-17/20 and C-18/19 (Pfister et al., 1996) are likely to be reverse.

MLCCC fraction II was rechromatographed using the analytical MLCCC device (160 m × 1.6 mm i.d. PTFE tubing) and *n*-BuOH/MeOH/H_2O (10:1:10) as the solvent system. From this separation, subfraction II.5 was acetylated and

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subjected to flash chromatography (Still et al., 1978). Final purification by normal phase HPLC on an Eurospher Si 100 column (5 μm, 250 × 4 mm, Knauer, Berlin, flow rate 1.0 mL/min, eluent: hexane/MTBE-gradient, UV detection 380 nm) yielded the peracetylated β-D-gentiobiosyl-β-D-glucopyranosyl ester of crocetin **3** (8.3 mg).

Spectral data of peracetylated **3**: ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.99 (6 H, d, J = 1.0 Hz, CH_3-C2/C15), 2.01 (6 H, s, CH_3-C6/C11), 2.02−2.09 (33 H, 11 acetates), 3.60 (1 H, dd, J = 11.5, 5.5 Hz, H_aC6′), 3.64 (1 H, ddd, J = 10.0, 4.5, 2.0 Hz, HC5″), 3.84 (1 H, ddd, J = 10.0, 5.5, 2.0 Hz, HC5′), 3.89 (1 H, ddd, J = 10.0, 4.5, 2.0 Hz, HC5°), 3.94 (1 H, dd, J = 11.5, 2.0 Hz, H_bC6′), 4.10 (1 H, dd, J = 12.0, 2.0 Hz, H_aC6″), 4.12 (1 H, dd, J = 12.5, 2.0 Hz, H_aC6°), 4.25 (1 H, dd, J = 12.0, 4.5 Hz, H_b-C6″), 4.32 (1 H, dd, J = 12.5, 4.5 Hz, H_bC6°), 4.56 (1 H, d, J = 8.0 Hz, HC1″), 4.99 (1 H, dd, J = 9.5, 8.0 Hz, HC2″), 5.02 (1 H, dd, J = 10.0, 9.5 Hz, HC4′), 5.06 (1 H, dd, J = 10.0, 9.5 Hz, HC4″), 5.17 (1 H, dd, J = 10.0, 9.5 Hz, HC4°), 5.19 (1 H, dd, J = 9.5, 9.5 Hz, HC3″), 5.21 (1 H, dd, J = 9.5, 8.0 Hz, HC2′), 5.28 (1 H, dd, J = 9.5, 9.5 Hz, HC3′), 5.28 (1 H, dd, J = 9.5, 8.0 Hz, HC2°), 5.30 (1 H, dd, J = 9.5, 9.5 Hz, HC3°), 5.75 (1 H, d, J = 8.0 Hz, HC1′), 5.78 (1 H, d, J = 8.0 Hz, HC1°), 6.43 (2 H, dd, J = 10.0, 1.0 Hz, HC7/HC10), 6.54 and 6.55 (2 × 1 H, dd, J = 15.0, 11.5 Hz, HC4/HC13), 6.68 and 6.69 (2 × 1 H, d, J = 15.0 Hz, HC5/HC12), 6.74 (2 H, m, HC8/HC9), 7.34 (2 H, dq, J = 11.5, 1.0 Hz, HC3/HC14); ^{13}C NMR (63 MHz, $CDCl_3$) δ 12.6 (CH_3-C2/C15), 12.8 (CH_3-C6/C11), 61.6 (C6°), 61.8 (C6″), 67.6 (C6′), 68.0 (C4°), 68.4 (C4″), 68.7 (C4′), 70.2 (C2′/C2°), 70.9 (C2″), 71.9 (C5″), 72.7 (C3°), 72.8 (C5°), 72.8 (C3′/C3″), 74.0 (C5′), 92.0 (C1′), 92.1 (C1°), 100.6 (C1″), 123.6 (C4/C13), 124.5 (C2/C15), 131.7 (C8/C9), 136.3 (C7/C10), 136.9 (C6/C11), 141.4 (C3), 141.4 (C14), 145.3 (C5/C12), 166.0 (C1), 166.1 (C16), 20.5−20.7 and 169.3−170.6 (11 acetates).

Isolation of (2*E*,4*E*)-2-Methyl-6-oxohepta-2,4-dienoic Acid β-D-Gentiobiosyl Ester 4. Acetylation of MLCCC subfraction II.1 followed by flash chromatography and final purification by RP-HPLC (Eurospher Si 100-C_{18} column, 5 μm, 250 × 16 mm, Knauer, Berlin, flow rate 10.0 mL/min, MeOH/H_2O-gradient, UV detection 320 nm) yielded the peracetylated gentiobiosyl ester **4** (5.3 mg): UV (MeOH) λ_{max} 283 nm; DCI-MS: pseudomolecular ion at m/z 790 $[M(772) + NH_4]^+$; ^{1}H NMR (360 MHz, $CDCl_3$) δ 2.00−2.08 (21 H, 7s, seven acetates), 2.06 (3 H, d, J = 1.5 Hz, CH_3-C2), 2.34 (3 H, s, H_3C7), 3.59 (1 H, dd, J = 11.5, 5.5 Hz, H_aC6′), 3.65 (1 H, ddd, J = 9.5, 4.5, 2.5 Hz, HC5″), 3.84 (1 H, ddd, J = 10.0, 5.5, 2.5 Hz, HC5′), 3.95 (1 H, dd, J = 11.5, 2.5 Hz, H_bC6′), 4.11 (1 H, dd, J = 12.5, 2.5 Hz, H_aC6″), 4.25 (1 H, dd, J = 12.5, 4.5 Hz, H_bC6″), 4.54 (1 H, d, J = 8.0 Hz, HC1″), 4.99 (1 H, dd, J = 9.5, 8.0 Hz, HC2″), 5.02 (1 H, dd, J = 10.0, 9.5 Hz, HC4′), 5.06 (1 H, dd, J = 9.5, 9.5 Hz, HC4″), 5.18 (1 H, dd, J = 9.5, 9.5 Hz, HC3″), 5.19 (1 H, dd, J = 9.5, 8.0 Hz, HC2′), 5.28 (1 H, dd, J = 9.5, 9.5 Hz, HC3′), 5.74 (1 H, d, J = 8.0 Hz, HC1′), 6.49 (1 H, d, J = 15.0 Hz, HC5), 7.26 (1 H, dq, J = 11.5, 1.5 Hz, HC3), 7.39 (1 H, dd, J = 15.0, 11.5 Hz, HC4); ^{13}C NMR (63 MHz, $CDCl_3$) δ 13.2 (CH_3-C2), 29.4 (C7), 61.8 (C6″), 67.5 (C6′), 68.4 (C4″), 68.5 (C4′), 70.2 (C2′), 70.9 (C2″), 71.9 (C5″), 72.7 (C3′), 72.8 (C3″), 74.0 (C5′), 92.4 (C1′), 100.6 (C1″), 133.9 (C2), 135.7 (C4), 136.0 (C5), 137.4 (C3), 165.1 (C1), 197.7 (C6), 20.6−20.7 and 169.2−170.6 (seven acetates). The arrangement of the substituents at the double bond in the 2,3-position was evaluated using NOE-experiments (cf. Figure 2).

Isolation of 3,5,5-Trimethylcyclohexenone Derivatives 5−7. 4-Hydroxy-3,5,5-trimethylcyclohex-2-enone 4-*O*-β-D-glucopyranosides **5** and **6** were obtained from MLCCC subfraction II.2 after acetylation, flash chromatography, and final purification by normal phase HPLC on an Eurospher Si 100 column (5 μm, 250 × 4 mm, Knauer, Berlin), flow rate 1.0 mL/min, eluent: pentane/MTBE (30:70), UV detection 220 nm.

Spectral data of peracetylated *(4R)-4-hydroxy-3,5,5-trimethylcyclohex-2-enone 4-O-β-D-glucopyranoside* (**5**) (1.5 mg): UV (MeOH) λ_{max} 234 nm; DCI-MS pseudomolecular ion at m/z 502 $[M(484) + NH_4]^+$; ^{1}H NMR (360 MHz, $CDCl_3$) δ 0.97 and 1.08 (2 × 3 H, 2s, 2 CH_3-C5), 2.01 (3 H, d, J = 1.0 Hz, CH_3-C3), 2.02−2.09 (12 H, 4s, four acetates), 2.18 (1 H, d, J = 16.5 Hz, H_aC6), 2.35 (1 H, d, J = 16.5 Hz, H_bC6), 3.72 (1 H, ddd, J = 9.5, 4.0, 3.0 Hz, HC5′), 3.98 (1 H, br s, HC4), 4.22 (1 H, d, J = 4.0 Hz, H_aC6′), 4.23 (1 H, d, J = 3.0 Hz, H_bC6′), 4.66 (1 H, d, J = 8.0 Hz, HC1′), 5.07 (1 H, dd, J = 9.5, 8.0 Hz, HC2′), 5.10 (1 H, dd, J = 9.5, 9.5 Hz, HC4′), 5.24 (1 H, dd, J = 9.5, 9.5 Hz, HC3′), 5.86 (1 H, br s, HC2); ^{13}C NMR (90 MHz, $CDCl_3$) δ 21.8 (CH_3-C3), 26.8 and 29.7 (2 CH_3-C5), 39.1 (C5), 48.9 (C6), 61.9 (C6′), 68.5 (C4′), 71.7 (C2′), 71.8 (C5′), 72.7 (C3′), 84.3 (C4), 101.9 (C1′), 127.2 (C2), 161.6 (C3), 198.0 (C1), 20.6−20.7 and 169.0−170.5 (four acetates).

Spectral data of peracetylated *(4S)-4-hydroxy-3,5,5-trimethylcyclohex-2-enone 4-O-β-D-glucopyranoside* (**6**) (1.5 mg): UV (MeOH) λ_{max} 229 nm; DCI-MS pseudomolecular ion at m/z 502 $[M(484) + NH_4]^+$; ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.00 and 1.04 (2 × 3 H, 2s, 2 CH_3-C5), 2.00 (3 H, br s, CH_3-C3), 2.01−2.09 (12 H, 4 s, four acetates), 2.16 (1 H, d, J = 16.5 Hz, H_aC6), 2.36 (1 H, d, J = 16.5 Hz, H_bC6), 3.70 (1 H, ddd, J = 9.5, 4.5, 3.0 Hz, HC5′), 4.05 (1 H, s, HC4), 4.21 (1 H, d, J = 4.5 Hz, H_aC6′), 4.22 (1 H, d, J = 3.0 Hz, H_bC6′), 4.69 (1 H, d, J = 8.0 Hz, HC1′), 5.05 (1 H, dd, J = 9.5, 8.0 Hz, HC2′), 5.10 (1 H, dd, J = 9.5, 9.5 Hz, HC4′), 5.23 (1 H, dd, J = 9.5, 9.5 Hz, HC3′), 5.86 (1 H, br s, HC2); ^{13}C NMR (90 MHz, $CDCl_3$) δ 21.9 (CH_3-C3), 27.1 and 29.7 (2 CH_3-C5), 38.4 (C5), 49.0 (C6), 62.0 (C6′), 68.6 (C4′), 71.7 (C2′), 71.8 (C5′), 72.8 (C3′), 82.9 (C4), 100.3 (C1′), 127.5 (C2), 161.4 (C3), 198.3 (C1), 20.6−20.7 and 169.1−170.4 (four acetates).

(4S)-4-(Hydroxymethyl)-3,5,5-trimethylcyclohex-2-enone-β-D-glucopyranoside (**7**). Acetylation of MLCCC fraction III followed by flash chromatography as well as final purification by normal phase HPLC on two Eurospher Si 100 column connected in series (5 μm, 250 × 4 mm, Knauer, Berlin, flow rate 1.5 mL/min, eluent: MTBE/hexane-gradient, UV detection 230 nm) yielded peracetylated glucoside **7** (1.0 mg): UV (MeOH) λ_{max} 233 nm; DCI-MS pseudomolecular ion at m/z 516 $[M(498) + NH_4]^+$; ^{1}H (360 MHz, $CDCl_3$) δ 1.03 and 1.08 (2 × 3 H, 2s, 2 CH_3-C5), 1.98−2.09 (12 H, 4s, four acetates), 2.00 (3 H, br s, CH_3-C3), 2.02 (1 H, d, J = 17.0 Hz, H_aC6), 2.07 (1 H, dd, J = 4.5, 3.5 Hz, HC4), 2.50 (1 H, d, J = 17.0 Hz, H_bC6), 3.62 (1 H, dd, J = 10.0, 4.5 Hz, H_aC10), 3.68 (1 H, ddd, J = 9.5, 4.5, 2.5 Hz, HC5′), 4.11 (1 H, dd, J = 10.0, 3.5 Hz, H_b-C10), 4.16 (1 H, dd, J = 12.5, 2.5 Hz, H_aC6′), 4.25 (1 H, dd, J = 12.5, 4.5 Hz, H_bC6′), 4.45 (1 H, d, J = 8.0 Hz, HC1′), 4.97 (1 H, dd, J = 9.5, 8.0 Hz, HC2′), 5.08 (1 H, dd, J = 9.5, 9.5 Hz, HC4′), 5.18 (1 H, dd, J = 9.5, 9.5 Hz, HC3′), 5.90 (1 H, br s, HC2). ^{1}H (360 MHz, C_6D_6) δ 0.76 and 0.82 (2 × 3 H, 2s, 2 CH_3-C5), 1.56 (1 H, dd, J = 4.5, 3.5 Hz, HC4), 1.67−1.70 (12 H, 4s, four acetates), 1.78 (3 H, br s, CH_3-C3), 2.04 (1 H, d, J = 17.0 Hz, H_aC6), 2.51 (1 H, d, J = 17.0 Hz, H_bC6), 3.15 (1 H, dd, J = 10.5, 4.5 Hz, H_aC10), 3.26 (1 H, ddd, J = 9.5, 4.5, 2.0 Hz, HC5′), 3.81 (1H, dd, J = 10.5, 3.5 Hz, H_bC10), 4.03 (1 H, d, J = 8.0 Hz, HC1′), 4.08 (1 H, dd, H = 12.0, 2.0 Hz, H_aC6′), 4.23 (1 H, dd, J = 12.0, 4.5 Hz, H_bC6′), 5.20 (1H, dd, J = 9.5, 8.0 Hz, HC2′), 5.23 (1 H, dd, J = 9.5, 9.5 Hz, HC4′), 5.38 (1 H, dd, J = 9.5, 9.5 Hz, HC3′), 5.92 (1 H, s, HC2); ^{13}C NMR (90 MHz, $CDCl_3$) δ 23.6 (CH_3-C3), 26.9 and 28.9 (2 CH_3-C5), 35.3 (C5), 48.1 (C6), 50.9 (C4), 61.9 (C6′), 67.9 (C10), 68.5 (C4′), 71.0 (C2′), 71.9 (C5′), 72.6 (C3′), 100.8 (C1′), 127.5 (C2), 159.6 (C3), 199.2 (C1), 20.4−20.7 and 169.1−170.6 (four acetates). Signals were assigned on the basis of the ^{1}H−^{13}C COSY data.

Aglycon and Sugar Analysis. After deacetylation with 0.02 M NaOMe in MeOH (cf. Winterhalter et al., 1991), each of the glucosides **5**−**7** in 1 mL of H_2O was acidified with 1 drop of diluted acetic acid and incubated overnight (37 °C) with 5 mg of β-glucosidase (sweet almond emulsin, Serva). The liberated aglycons were extracted with Et_2O (2 × 2 mL) and analyzed by GC−MS. *4-Hydroxy-3,5,5-trimethylcyclohex-2-enone*: R_i (DB-5) 1309, R_i (DB-Wax) 1411; EI-MS (70 eV) m/z (%) 154 (M^+, 2), 139 (1), 112 (35), 98 (100), 70 (43), 69 (37), 55 (10), 43 (13), 42 (29), 41 (25). *4-(Hydroxymethyl)-3,5,5-trimethylcyclohex-2-enone*: R_i (DB-5) 1492, R_i (DB−Wax) 1370; EI-MS (70 eV) m/z (%) 168 (M^+, 28), 153 (6), 138 (8), 123 (100), 112 (33), 111 (54), 97 (29), 79 (20), 69 (25), 67 (36), 55 (21), 41 (37); sign of optical rotation: (−); CD (c = 0.01% in MeOH) $[\theta]_{336}$ −183, $[\theta]_{246}$ −1737, $[\theta]_{210}$ +3533. For additional spectral data, cf. Davis and Johnson (1979). Comparison of the CD data with those published for 3-oxo-α-ionone suggest *S*-configuration at C4 (Schreier, 1993). The aqueous layer was passed through an ultrafilter (Ultrafree-MC 5000 NMGG, Millipore) and 20 μL of the enzyme-free filtrate was then injected into the HPLC system (Shandon Hypersil APS-5 μm

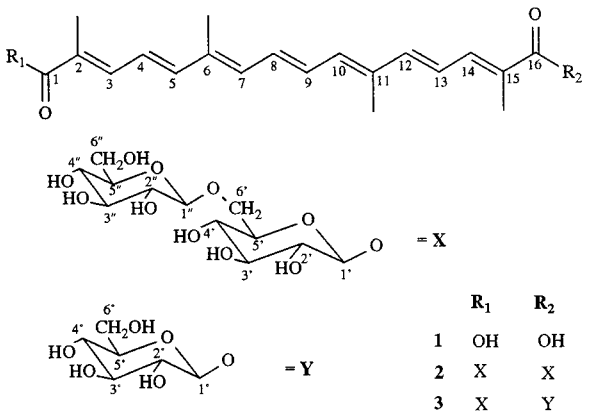

Figure 1. Structures of two major saffron pigments separated by preparative MLCCC (for details, cf. text).

column, 125 × 4.6 mm; eluent:acetonitrile/H_2O, 80:20). The presence of D-glucose was verified by on-line coupled refractive index (RI detector, Knauer, Berlin) and polarimetric detection (Chiralyzer polarimetric detector, IBZ Messtechnik, Hannover).

High-Resolution Gas Chromatography (HRGC). Dani educational gas chromatographs equipped with either a J&W fused silica DB-5 capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm) or a J&W fused silica DB-Wax capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm) were used. Split injection (1:20) was employed. The temperature program was from 60 °C (2 min isothermal) to 300 °C at 5 °C/min for DB-5 and from 50 °C (3 min isothermal) to 215 °C at 4 °C/min for DB-Wax, respectively. The flow rate for the carrier gas was 1.5 mL/min of He, for the makeup gas 30 mL/min of N_2, and for the detector gases 37 mL/min of H_2 and 280 mL/min of air. The injector temperature was kept at 250 °C and the detector temperature at 280 °C for DB-5; these temperatures for DB-Wax were 220 and 250 °C, respectively. The linear retention index (R_i) is based on a series of *n*-hydrocarbons.

High-Resolution Gas Chromatograpy−Mass Spectrometry (HRGC−MS). HRGC−MS was performed with a Hewlett-Packard GCD system equipped with a PTV injector (KAS-system, Gerstel, Mülheim, Germany). The same types of columns and the same temperature programs as mentioned above for HRGC analysis were used. Other conditions were as follows: carrier gas flow rate, 1.2 mL/min of He; temperature of ion source, 180 °C; electron energy, 70 eV; injection volumes, 1 μL.

Nuclear Magnetic Resonance (NMR). ^1H and ^{13}C NMR spectral data were recorded on Fourier transform Bruker AM 360 and AC 250 spectrometers with TMS as the internal reference standard. Signals were assigned by $^1H-^1H$ COSY and $^1H-^{13}C$ COSY, as well as HMBC experiments.

Desorption Chemical Ionization Mass Spectrometry (DCI-MS). DCI-MS was carried out with a Finnigan TSQ 70 mass spectrometer at 70 eV using ammonia as the reactant gas and ion source temperature and pressure of 150 °C and 1.5×10^{-4} mbar, respectively, as well as a temperature gradient of 400 °C/min. Mass range was 60−900.

Circular Dichroism (CD). CD spectra were recorded in MeOH (20 °C) using a JASCO J-710 polarimeter.

RESULTS AND DISCUSSION

A methanolic extract of saffron was fractionated with the aid of multilayer coil countercurrent chromatography (MLCCC) using $CHCl_3$/MeOH/H_2O (7:13:8) as the solvent system. The so-obtained subfractions I−V were rechromatographed using the analytical MLCCC device and *n*-BuOH/MeOH/H_2O (10:1:10) as the solvent system. The separated fractions were acetylated and further purified by preparative and/or analytical HPLC. Besides known saffron pigments, i.e., the digentiobiosyl- and gentiobiosylglucosyl esters of crocetin (cf. Figure 1), which were clearly separated by MLCCC, four novel

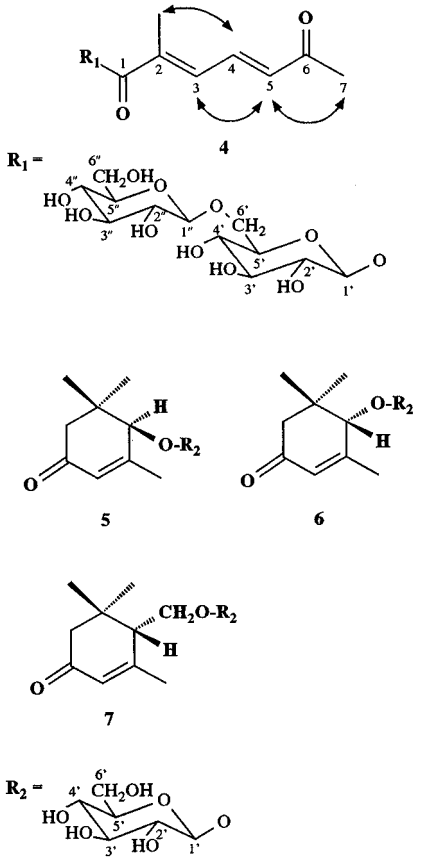

Figure 2. Structures of novel glucoconjugated apocarotenoids **4**−**7** from saffron (arrows indicate NOEs).

glycosidic constituents were obtained in minor amounts (cf. Figure 2). A monogentiobiosyl ester—structurally related to crocin **2**—was isolated from MLCCC fraction I. The proton spectrum included all the signals typical for the acetylated gentiobiosyl moiety (Gagnaire et al., 1976; Güldner and Winterhalter, 1991), as well as a three-proton singlet at δ 2.34 ppm (methyl ketone) and a three-proton doublet (δ 2.06 ppm, J = 1.5 Hz, vinyl methyl) long-range coupled to an olefinic system consisting of a trisubstituted as well as an *E*-configured double bond. From the carbon spectrum, a carboxyl (δ 165.1 ppm) as well as a carbonyl function (δ 197.7 ppm) was apparent together with four olefinic carbons and two methyl groups. The structure **4** assigned to this gentiobiosyl ester was further supported by the recorded DCI-MS spectrum which showed a strong pseudomolecular ion at *m*/*z* 790 $[M + NH_4]^+$ as well as its UV data. The formation of this novel gentiobiosyl ester can be rationalized by an oxidative cleavage of the zeaxanthin chain at the double bonds located in positions 7,8 and 13,14, respectively.

In addition to carotenoid metabolite **4**, glycoconjugates bearing a C_9 or C_{10} carotenoid end group as aglycon moiety have also been detected. Compounds **5** and **6** were found to be the diastereoisomeric β-D-glucopyranosides of 4-hydroxy-3,5,5-trimethylcyclohex-2-enone which could be separated by analytical HPLC. The coupling constant of the anomeric proton (J = 8.0 Hz) and the other signals of the glycon moiety were in good agreement with those published for acetylated β-D-glucopyranosides (Gagnaire et al., 1976). The aglycon moiety of **5** showed two three-proton singlets at δ 0.97 and 1.08 ppm (*gem*-dimethyl), a three proton doublet at δ 2.01 ppm (J = 1 Hz, vinyl methyl) long-range coupled to an olefinic proton (δ 5.86 ppm), and an AB-system centered at δ 2.26 ppm as well as a one-proton

singlet at δ 3.98 ppm (hydroxymethine). For the diastereomer **6**, a similar pattern of signals with slightly different chemical shift frequencies was observed, thus suggesting the C_9-norisoprenoid 4-hydroxy-3,5,5-trimethylcyclohex-2-enone as the aglycon moiety. The proposed structure was confirmed by the ^{13}C NMR, DCI-MS, and UV data. With regard to the configuration at C4, enzymatic hydrolyses of the deacetylated glycoconjugates have been carried out (Skouroumounis and Winterhalter, 1994). β-Glucosidase treatment of **6** liberated the levorotatory isomer of 4-hydroxy-3,5,5-trimethylcyclohex-2-enone, whereas in the case of glucoside **5,** the dextrorotatory form was obtained. On the basis of these results, the configuration of the chiral center C4 was assigned as *S* in structure **6** and *R* in structure **5** (Mikami et al., 1981).

A further glucopyranoside showing a similar trimethylcyclohexene skeleton was isolated from MLCCC fraction III. From the proton NMR, however, the presence of an additional hydroxymethylene group was apparent. Structure **7** assigned to this C_{10}-glucoside is in agreement with the recorded ^{13}C NMR, DCI-MS, and UV data. CD data recorded for the aglycon moiety suggest *S*-configuration at the chiral center C4.

Glycoconjugates **4**−**7** are to our best knowledge reported here for the first time as natural products. The aglycon moiety of glucosides **5** and **6**, however, has earlier been reported as free aroma constituent of saffron (Zarghami and Heinz, 1971; Rödel and Petrzika, 1991). With regard to the bio-oxidative cleavage of saffron carotenoids, the detection of structures **4**−**7** suggests that, in addition to the assumed 7,8-carotenase cleavage of the polyene chain, other cleavage reactions have also to be considered. The question whether or not the formation of carotenoid-breakdown products **4**−**7** is enzyme-mediated or due to other oxidative processes, must be addressed in ongoing studies.

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