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BIOSYNTHESIS OF C_{20}-CAROTENOIDS IN *CROCUS SATIVUS*

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Key Word Index—*Crocus sativus*; Iridaceae; saffron; biosynthesis; carotenoids; phytoene; phytofluene; tetrahydrolycopene; β-carotene; zeaxanthin; crocetin.

Abstract—Phytoene, phytofluene, tetrahydrolycopene, β-carotene, zeaxanthin and crocetin were isolated from *Crocus sativus*. The absence of C_{20}-hydrocarbon precursors of crocetin supports a degradation pathway for the biosynthesis of crocetin.

INTRODUCTION

The main pigments of saffron (stigma of *Crocus sativus* L.) are mono- and diglycosyl esters of the polyene dicarboxylic acid crocetin (**1**, $R_1 = R_2 = H$) whereby D-glucose and D-gentiobiose occur as carbohydrate residues [1–3]. In addition to these crocetin derivatives minor amounts of β-carotene (β,β-carotene), γ-carotene (β,Ψ-carotene), lycopene (Ψ,Ψ-carotene) and zeaxanthin (**2**,β,β-carotene-3,3′-diol) have been isolated. Methodical investigations of the biosynthesis of these polyenes in saffron have not been carried out previously. In principle, two different pathways for the biosynthesis of the C_{20}-aglycone (**1**) of the main pigments are possible: (a) an oxidative degradation of a C_{40}-carotenoid such as zeaxanthin (**2**) as suggested earlier [4], or (b) a dimerization of two C_{10}-compounds, such as geranylpyrophosphate (**3**), followed by dehydrogenation and oxidation in analogy to the formation of C_{30}-carotenoids from farnesylpyrophosphate (C_{15}) in *Staphylococcus aureus* and *Streptococcus faecium* [5] and the formation of C_{40}-carotenoids from geranylgeranylpyrophosphate (C_{20}) [6] (see Scheme 1).

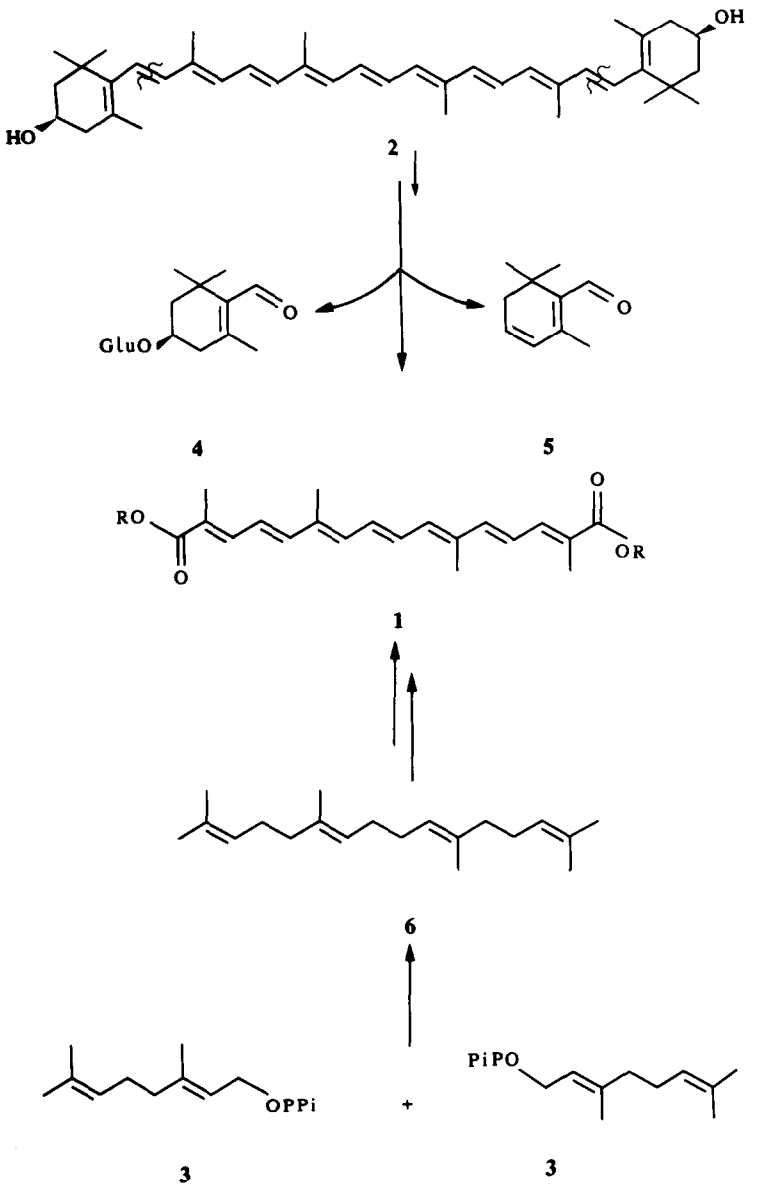
A degradation pathway is supported by the occurrence of picrocrocin (**4**) and safranal (**5**) [4], the former having the same stereochemistry as zeaxanthin [7, 8]. On the other hand no strict evidence has been given for this pathway and one can as easily assume a dimerization of geranylpyrophosphate (**3**), in which case the C_{20}-analogues (**6–9**) of lycopersene, phytoene, phytofluene and ζ-carotene should be present in saffron. In order to investigate this second possibility the four hydrocarbons **6–9** were synthesized as reference compounds [9].

In the present paper we report the isolation of carotenoid precursors from saffron.

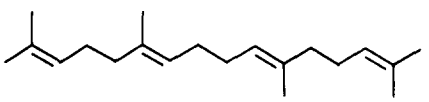
RESULTS AND DISCUSSION

The ether extract of saffron (2 kg) was separated by repeated CC (Si gel, solvents A and B) into five fractions A–E. The least polar fraction A, which could not be saponified, should contain the possible C_{20}-hydrocarbons. Comparative HPLC (LiChrosorb SI 60, 5 μm, solvent C) however revealed the absence of compounds **6–9** but indicated the presence of less polar compounds [10]. Phytofluene (**10**) was separated from this mixture by CC (Al_2O_3, solvent D) and was identified by its UV spectrum (λ_{max}^{MeCN} nm: 330, 348, 368) and its mass spectral fragmentation pattern: *m/z* 542 (M^+ for $C_{40}H_{52}$), 474 $[M-69+H]^+$, 406 $[M-137+H]^+$ and 337 $[M-205]^+$. The remaining three components of fraction A were separated by repeated HPLC (ODS, solvent E) and identified as tetrahydrolycopene (**11**) ($k' = 3.5$), β-carotene (**12**) ($k' = 3.93$), and phytoene (**13**) ($k' = 5.78$), respectively. Compound **11** showed the UV spectrum of a conjugated heptaene (λ_{max}^{MeCN} nm: 376, 397, 421) and mass spectral fragments at *m/z* 472 $[M-69+H]^+$, 403 $[M-137]^+$ and 305 $[M-205]^+$. Though the fragment of *m/z* 335 $[M-205]^+$ suggested a break of the bisallylic 11,12-bond of 7,8,11,12-tetrahydrolycopene no corresponding metastable peak at *m/z* 208 ($335^2/540 = 207.8$) could be found [11]. The alternative structure of the symmetrical ζ-carotene also can show a peak at *m/z* 335 $[M-137-69+H]^+$ which should result in a metastable peak at *m/z* 278 ($335^2/403 = 278.5$) or at 238 ($335^2/472 = 237.8$). No such metastable peak could be found which is not surprising since the corresponding parent ions have very low intensities. We therefore cannot differentiate between the two structures. However, in higher plants only the symmetrical ζ-carotene is normally formed and we therefore assume its structure for compound **11**. Compound **12** was identified by its k'-value [10] and its spectroscopic data (UV and mass spectrum) [12]. Compound **13** had the UV spectrum of a conjugated triene (λ_{max}^{MeCN} nm: 276, 286, 297) and mass spectral peaks at *m/z* 544 (M^+ for $C_{40}H_{44}$), 406 $[M-137+H]^+$ and 339 $[M-205]^+$.

After saponification of fractions B and C and subsequent chromatography (prep. TLC, solvent F, and HPLC, ODS, solvent G for fraction B; HPLC, ODS,



Scheme 1. Possible pathways for the formation of crocetin derivatives.



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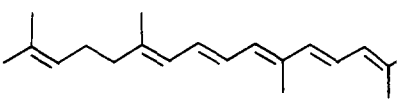

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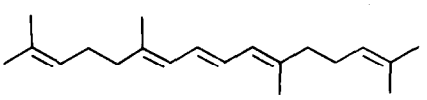

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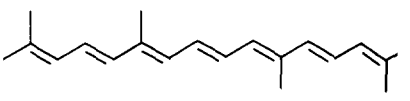

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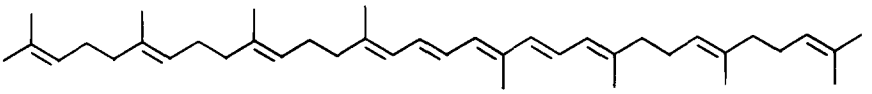

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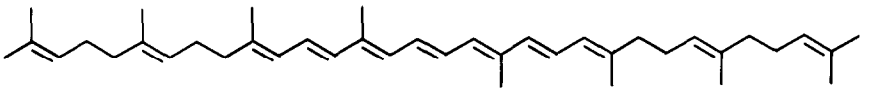

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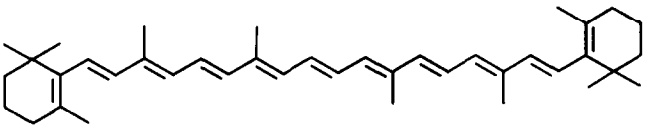

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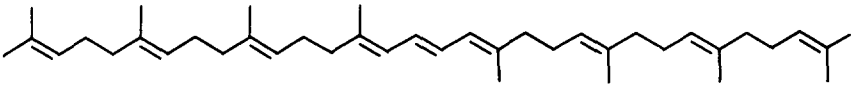

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solvents H and G for fraction C) *cis*- and all-*trans* isomers, respectively, of zeaxanthin (**2**) could be identified by UV, ^{1}H NMR (270 MHz) and mass spectrometry [12]. Saponification of fractions D and E was used as a purification step but no saponification of the pigments themselves took place. Fraction D gave all-*trans* zeaxanthin (**2**) which was crystallized from ethanol after prep. TLC (Si gel, solvents A and I). Its UV, ^{1}H NMR (270 MHz) and mass spectra were identical with those of synthetic zeaxanthin [12]. Fraction E finally furnished crystalline crocetin (**1**, $R_1 = R_2 = H$) (from DMF) after prep. TLC (Si gel, solvent K). Its UV, ^{1}H NMR (270 MHz) and mass spectra were identical with those of synthetic crocetin [12]. This is the first time that free **1** and **2** have been isolated from saffron without previous saponification of the whole extract.

Although we had full knowledge of the chromatographic and spectroscopic behaviour of the possible crocetin precursors **6–9**, no such hydrocarbon could be found in saffron. However, this is the first time that the C_{40}-hydrocarbons **10**, **11** and **13**, which are known precursors of β-carotene (**12**), and the xanthophylls **1** ($R_1 = R_2 = H$) and **2** have been found in saffron. These results support a degradation pathway for the biosynthesis of crocetin and the dimerization of geranylpyrophosphate (**3**) can most probably be excluded. Only the possibility of an early hydroxylation before the dehydrogenation steps is still open.

In contrast to earlier investigations no γ-carotene or lycopene were found in our saffron. Possibly the carotenoid composition of saffron varies with age and origin of the sample. Further studies on the pathway of biosynthesis of the crocetin derivatives are now in progress.

EXPERIMENTAL

Safran electus pulvis (selected and pulverized saffron) commercially available from Siegfried Ltd., Zofingen, Switzerland, was used.

Chromatography. Si gel (63–200 μm) and Al_2O_3 (activity II–III) partion chromatography was carried out in columns of various sizes. For prep. TLC pre-coated plates (Si gel 60 F 254, layer thickness 2 mm) were used. HPLC conditions were reported earlier [10], ODS: octadecylsilane. Solvents: A, petrol–toluene–EtOH (5:5:1); B, toluene–*n*-hexane (1:1); C, *n*-pentane; D, *n*-hexane; E, MeCN; F, petrol–THF (2:1); G, MeOH–MeCN (1:1); H, MeOH–MeCN (7:3); I, $CHCl_3$–Me_2CO (4:1); K, $CHCl_3$–Me_2CN–dioxane (13:3:4).

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