Isolation and Structure Elucidation of Two (*Z*)-Isomers of Lutein from the Petals of Rape (*Brassica napus*)

Daniel R. Kull and Hanspeter Pfander*

Department of Chemistry and Biochemistry, University of Berne, Freiestrasse 3, CH-3012 Berne, Switzerland

The yellow petals of rape (*Brassica napus*) were extracted with hexane/acetone/MeOH/toluene (10:7:6:7), EtOAc/hexane/acetone (6:1:1), and EtOAc/Et_2O (1:1), and subsequently the saponified extract was submitted to a step gradient on an Al_2O_3 column. From the most polar fraction, two (*Z*)-isomers of lutein have been isolated by reversed-phase HPLC. On the basis of UV−vis, CD, 1D and 2D 400 MHz ^{1}H-NMR, and mass spectra they were identified as (9*Z*,9′*Z*,3*R*,3′*R*,6′*R*)-lutein and (13′*Z*, 3*R*,3′*R*,6′*R*)-lutein.

Keywords: *Carotenoids; isolation; structure elucidation; Brassica napus; (Z)-isomers of lutein*

INTRODUCTION

Rape (*Brassica napus*) belongs to the family of the Cruciferae and is botanically closely related to other *Brassica* species like cabbage, cauliflower, and mustard. The name *crucifer* originates from the arrangement of the plants' flower petals: diagonally opposite each other in the form of a cross. Rape is widely cultivated in temperate regions of the northern hemisphere, like Asia, throughout Europe, and in Canada, as a forage crop for livestock and as a cover crop. The seeds of the rape plant are valued for their yellowish oil, which can be used, *e.g.*, for cooking, as a lubricant, and for making soap (Shahidi, 1990; Kull and Pfander, 1995).

Previous HPLC investigations exhibited the occurrence of more than 80 different carotenoids in the petals of rape, ranging from hydrocarbons to highly oxygenated compounds, including naturally occurring (*E*/*Z*)-isomers (Kull and Pfander, 1995).

Recently we reported the isolation of the major polar pigments from the petals of rape, which have been identified as (9′*Z*)-neoxanthin, (8′*R*)- and (8′*S*)-luteoxanthin, (13*Z*,8′*R*)-luteoxanthin, (*all-E*)-, (9*Z*)-, and (13*Z*)-violaxanthin, taraxanthin, and a 1:1 mixture of flavoxanthin and chrysanthemaxanthin (Debrunner and Pfander, 1991; Kull, 1996).

In continuation of this work, we report now the isolation and structure elucidation of two additional carotenoids from the most polar fraction, which were identified as (*Z*)-isomers of lutein.

MATERIALS AND METHODS

For information concerning plant material and special precautions, please refer to our previous publication (Kull and Pfander, 1995).

General Procedures. Column chromatography: "flash" column (∅ 5 cm); neutral Al_2O_3III, obtained from neutral Al_2O_3 I (Camag, Muttenz) by addition of 6% H_2O and stirring for 12 h. Lobar system: Sepapress PCP-150/75 pressure ceramic pump; Merck Lobar column, size B (310-25), Lichroprep RP-8, 40−63 μm; Uvikon LCD 725 detector. HPLC was performed with a Waters 600E multisolvent delivery system (Laboc Gastorr GT-104 (4-channel online degasser); Rheodyne 7725i injector), connected to a Waters PDA 996 photodiode array detector; data processing was carried out by Millennium 2010 software. UV−vis spectra were measured on a Perkin-Elmer 554 photospectrometer or on the Waters PDA 996, λ_{max} in nm. The fast atom bombardment (FAB) mass spectra were recorded on a VG Autospec instrument. The circular dichroism (CD) spectra were measured on a Dichrograph CD6 (Jobin-Yvon) with MeOH as solvent. The ^{1}H-NMR spectra were recorded on a Bruker AM 400 (400 MHz) spectrometer; $CDCl_3$ was used as solvent with TMS as internal standard.

Extraction and Isolation. A detailed description of the general extraction and isolation procedures has been given in Kull and Pfander (1995). The sharp red band which was eluted with MeOH as the most polar fraction from the Al_2O_3 column was further separated on a C_8-Lobar column. For isocratic elution a solvent mixture of MeOH/H_2O/EtOAc (88.5:8.5:3) with addition of 1% triethylamine was used. The column pressure during the separation was about 3 bar, which corresponds to a flow of 5.5 mL/min. This value was chosen because during sample loading the pressure rose to 8 bar and the pressure limit of the glass column of 10 bar was not to be exceeded.

The three main peaks with the known carotenoid structures were eluted between 11 and 45 min. The peak to be investigated had a retention time of 65 min and was collected from about 58 min until the absorption reached the base line. As an isocratic system was used, periodic injections every 70 min could be made without loss of the quality of the collected fraction.

HPLC Separation. For the HPLC separation of the last fraction of the Lobar separation a reversed-phase column (250 × 10 mm Nucleosil 7 C_{18}; Macherey-Nagel, Switzerland), developed with 2-propanol/H_2O (85:15), at a flow rate of 2 mL/min, was used. Besides some impurities with t_R > 12 min, two main peaks with (9*Z*,9′*Z*)-lutein (t_R = 13.1 min) and (13′*Z*)-lutein (t_R = 14.5 min) could be isolated. Both fractions were, after evaporation of the solvent, submitted a second time to the same chromatographic system to remove impurities. A second purity check was performed on the same column with 100% MeOH. At a flow rate of 2 mL/min, retention times of 16 min for (9*Z*,9′*Z*)-lutein and 23 min for (13′*Z*)-lutein were measured. There were no impurities or isomerization products observed.

RESULTS AND DISCUSSION

The whole extract of the lyophilized petals of rape was separated by column chromatography (CC) with Al_2O_3 to give six fractions containing carotenoids of different polarities (Debrunner and Pfander, 1991; Kull and Pfander, 1995). The most polar fraction appeared on the column as a sharp red band and was eluted with

* Author to whom correspondence should be addressed (phone +41 31 631 4382; fax +41 31 631 3425; e-mail pfander@ioc.unibe.ch).

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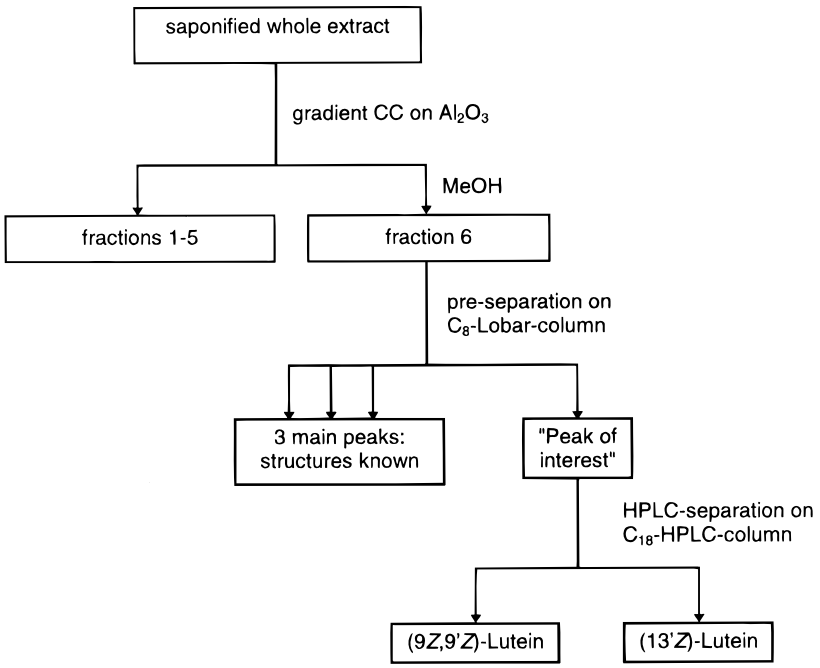
Scheme 1. Isolation Procedure Employed for the Isolation of (9*Z*,9′*Z*)- and (13′*Z*)-Lutein



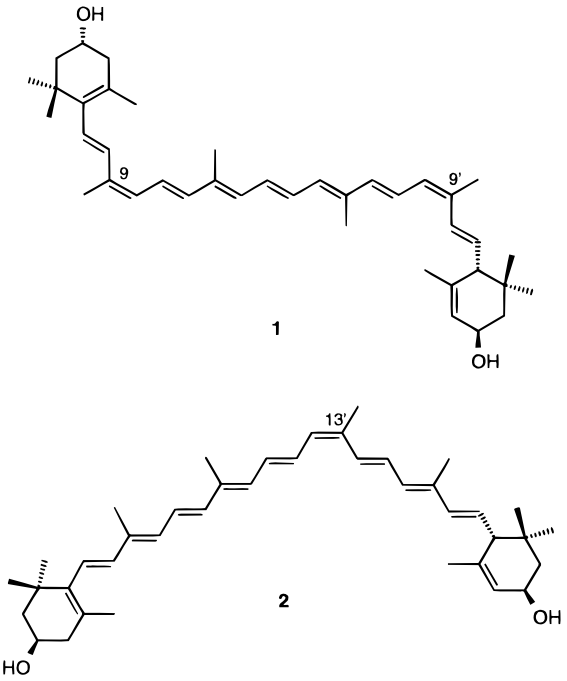


Figure 1. Structures of (9*Z*,9′*Z*,3*R*,3′*R*,6′*R*)-lutein (**1**) and (13′*Z*,3*R*,3′*R*,6′*R*)-lutein (**2**).

MeOH. Afterward this fraction was submitted to CC with a C_8-Lobar column. Besides three peaks which contained the carotenoids previously identified, an additional peak was observed. The further separation was carried out by semipreparative HPLC on a C_{18} column with 2-propanol/H_2O (85:15) as eluent. (See Scheme 1.)

From the first peak of the HPLC separation a total amount of 4.5 mg of a carotenoid with a retention time of 13.1 min was isolated, which on the basis of the UV−vis, CD, ^{1}H-NMR, and mass spectra was identified as (9*Z*,9′*Z*,3*R*,3′*R*,6′*R*)-lutein (**1**) (Figure 1).

In the UV−vis spectrum two main absorption bands at 470 and 442 nm and a shoulder at 420 nm were observed. These λ_{max} values are consistent with a polyene chain of 10 conjugated double bonds. In comparison with (*all-E*)-lutein the absorption maxima were shifted for 5 nm toward shorter wavelengths, which is typical for (*Z*)-isomers. Only a small "cis-peak" at 331 nm, approximately 140 nm below the absorption with the longest wavelength, was observed, which gave an indication that the isolated compound did not correspond to the (13*Z*)/(13′*Z*)- or (15*Z*)-isomer (Britton, 1995).

The FAB mass spectrum showed the molecular ion at *m*/*z* 568, which is in agreement with the molecular formula $C_{40}H_{56}O_2$ of lutein. Furthermore typical fragments for the hydroxylated β- and ϵ end groups were observed. The signals at *m*/*z* M^+-18, M^+-56, M^+-138, and M^+-153 are characteristic for the hydroxylated ϵ end group and those at *m*/*z* M^+-18, M^+-153, and M^+-193 for the hydroxylated β end group (Enzell and Back, 1995).

The data of the 1D and 2D ^{1}H-NMR spectra and the comparison with literature confirmed the presence of the 3-hydroxy β end group and the 3-hydroxy ϵ end group (Englert, 1995). Compared to (*all-E*)-lutein, shifts toward lower field for H−C(7) and H−C(7′) of 0.54 ppm and for H−C(11) and H−C(11′) of 0.1 ppm were observed. In contrast the signals of H−C(10) and H−C(10′) as well as those of H−C(12) and H−C(12′) were shifted to higher field by 0.1 and 0.05 ppm, respectively. These data are characteristic for (9*Z*)-isomers, and therefore the isolated compound was identified as (9*Z*,9′*Z*)-lutein.

The determination of the absolute configuration at C(3), C(3′) and C(6′) in lutein was carried out by CD. The data was in accordance with the literature for (9*Z*,9′*Z*,3*R*,3′*R*,6′*R*)-lutein (**1**) (Buchecker and Noack, 1995).

The second peak of the HPLC separation with a retention time of 14.6 min afforded after chromatography 3.2 mg of carotenoid **2**. On the basis of the UV−vis, CD, ^{1}H-NMR, and mass spectra the carotenoid **2** was identified as (13′*Z*,3*R*,3′*R*,6′*R*)-lutein.

The UV−vis spectra of **2** were similar to those of **1**. The main absorption maxima (467 nm, 440 nm) were shifted by ca. 8 nm toward lower wavelengths compared to the absorptions of (*all-E*)-lutein. Furthermore at 331

nm, ca. 140 nm below the absorption with the longest wavelength, a "cis peak" of significant intensity was observed. This gives an indication that in **2** the *(Z)*-double bond is located in the middle part of the molecule.

The FAB-MS measurements showed the molecular ion at m/z 568, which was in accordance with the molecular formula $C_{40}H_{56}O_2$ for lutein. The fragmentation was very similar compared with the spectrum of **1**, confirming the hydroxylated β end group and the hydroxylated ϵ end group.

In the 1D and 2D ^{1}H-NMR spectra a doubling of certain signals in the olefinic region was observed, indicating an unsymmetrical position of the (*Z*)-double bonds of the polyene chain. Compared to the signals of the (*all-E*)-isomer of lutein, a shifts toward lower field were observed for the signal H−C(10′) of 0.05 ppm, for H−C(12′) of 0.52 ppm, and for H−C(15′) of 0.16 ppm. At the same time the signals of H−C(14′) and H−C(15′) were shifted toward higher field by 0.16 and 0.08 ppm, respectively. These shifts are characteristic for (13′*Z*)-isomers, and therefore **2** was identified as (13′*Z*)-lutein (Englert, 1995).

The determination of the absolute configuration at C(3), C(3′) and C(6′) in lutein was carried out by CD. The data was in accordance with the literature for (13′*Z*,3*R*,3′*R*,6′*R*)-lutein (**2**) (Buchecker and Noack, 1995).

(13′*Z*)-Lutein (**2**) was previously isolated from human plasma, and it can be assumed that this geometrical isomer is a metabolite of dietary (*all-E*)-lutein (Khachik, 1990).

To our knowledge it is the first time that (9*Z*,9′*Z*)-lutein (**1**) has been isolated and identified from natural sources. Due to the fact that (9*Z*,9′*Z*)-lutein (**1**) was found in significant amounts, while (9*Z*)-lutein was only present as a minor compound, we consider (9*Z*,9′*Z*)-lutein (**1**) not to be an artifact or an isomerization product but a naturally occurring carotenoid.

It is the first time that these two (*Z*)-isomers of lutein were found in plant tissue.

Characterization of the Carotenoids. *(9Z,9′Z)-Lutein [(9Z,9′Z,3R,3′R,6′R)-β,ϵ-Carotene-3,3′-diol] (1).* UV−vis: λ_{max} (nm) 470, 442, 420 (shoulder), 331 (2-propanol/H_2O 85:15); 472, 444, 422 (shoulder), 333 A_B/A_{II} = 8.8 (MeOH). CD: 217.5 (10.4), 242.5 (−13.5), 278.0 (3.6), 344.5 (−5.4), 452.5 (5.9), 471.5 (3.3), 484.5 (6.7). ^{1}H-NMR: 0.86 (s, 3H, CH_3(16′)), 1.03 (s, 3H, CH_3(17′)), 1.08, (s, 3H, CH_3(16)), 1.09 (s, 3H, CH_3(17)), 1.36 (m, br, 1H, H−O(3)), 1.37 (m, 1H, H_{eq}−C(2′)), 1.50 (m, 1H, H_{ax}−C(2)), 1.65 (s, 3H, CH_3(18′)), 1.78 (s, 3H, CH_3(18)), 1.78 (m, 1H, H_{eq}−C(2)), 1.85 (m, 1H, H_{ax}−C(2′)), 1.91 (s, 3H, CH_3(19′)), 1.96 (s, 3H, CH_3(20′)), 1.97 (s, 3H, CH_3(19)), 1.97 (s, 3H, CH_3(20)), 2.05 (m, 1H, H_{ax}−C(4)), 2.42 (m, 1H, H_{eq}−C(4)), 2.47 (d, 1H, H−C(6′)), 4.03 (m, br, 1H, H_{ax}−C(3)), 4.26 (m, br, 1H, H_{ax}−C(3′)), 5.46 (d, 1H, H−C(7′)), 5.55 (m, 1H, H−C(4′)), 6.03 (d, 1H, H−C(10′)), 6.07 (d, 1H, H−C(10)), 6.12 (d, 1H, H−C(7)), 6.25 (m, 2H, H−C(14/14′)), 6.30 (d, 2H, H−C(12/12′)), 6.67 (d, 2H, H−C(8/8′)), 6.63 (m, 2H, H−C(15/15′)), 6.73 (dd, 1H, H−C(11)), 6.74 (dd, 1H, H−C(11′)). MS: 568 (M^+, 24), 550 (M^+−18, 20), 540 (M^+−28, 8), 518 (100), 476 (3), 462 (6), 430 (21), 422 (19), 404 (6), 369 (12), 338 (8), 314 (7), 106, 92.

(13′Z)-Lutein [(13′Z,3R,3′R,6′R)-β,ϵ-Carotene-3,3′-diol] (2). UV−vis: 467, 440, 417 (shoulder), 331 (2-propanol/H_2O 85:15); 469, 442, 419 (shoulder), 333 A_B/A_{II} = 47.5 (MeOH). CD: 216.5 (20.2), 242.5 (−14.2), 279.0 (16.6), 330.0 (−14.0), 335.0 (−12.9), 344 (−19.8), 428.5 (6.5), 439.5 (5.0), 454.5 (9.15), 471.5 (4.6), 483.0 (9.8). ^{1}H-NMR: 0.85 (s, 3H, CH_3(16′)), 1.00 (s, 3H, CH_3(17′)), 1.07 (s, 3H, CH_3(16)), 1.08 (s, 3H, CH_3(17)), 1.37 (m, 1H, H_{eq}−C(2′)), 1.50 (m, 1H, H_{ax}−C(2)), 1.63 (s, 3H, CH_3(18′)), 1.77 (s, 3H, CH_3(18)), 1.78 (m, 1H, H_{eq}−C(2)), 1.85 (m, 1H, H_{ax}−C(2′)), 1.91 (s, 3H, CH_3(19′)), 1.93 (s, 3H, CH_3(19)), 1.96 (s, 3H, CH_3(20′)), 1.97 (s, 3H, CH_3(20)) 2.06 (m, 1H, H_{ax}−C(4)), 2.38 (d, 1H, H−C(6′)), 2.40 (m, 1H, H_{eq}−C(4)), 4.00 (m, br, 1H, H_{ax}−C(3)), 4.25 (m, br, 1H, H_{ax}−C(3′)), 5.43 (d, 1H, H−C(7′)), 5.55 (m, 1H, H−C(4′)), 6.02 (d, 1H, H−C(14′)), 6.08 (d, 1H, H−C(10)), 6.10 (d, 1H, H−C(7)), 6.17 (m, 1H, H−C(14)), 6.18 (d, 1H, H−C(10′)), 6.28 (d, 1H, H−C(12)), 6.09 (d, 1H, H−C(8′)), 6.15 (d, 1H, H−C(8)), 6.48 (m, 1H, H−C(15)), 6.54 (dd, 1H, H−C(11)), 6.58 (dd, 1H, H−C(11′)), 6.72 (d, 1H, H−C(15′)), 6.82 (d, 1H, H−C(12′)). MS: 582 (M^+ + CH_3, 23), 568 (M^+, 16), 550 (M^+−18, 7), 543 (M^+−28, 9), 532 (5), 517 (100), 462 (3), 447(12), 430 (44), 421 (31), 416 (5), 404 (6), 391 (8), 373 (9), 359 (6), 327 (7), 312 (12), 308 (17), 106, 92.

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