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SEMI-β-CAROTENONE FROM LEAVES OF TWO CYCADS

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Abstract—A rare secocarotenoid, semi-β-carotenone, has been isolated from young leaves of *Ceratozamia fuscoviridis* (a form of *C. mexicana* Brongn.) and of *C. kuesteriana*. This is the first time that this carotenoid has been obtained from photosynthetic tissue. New data on the chromatographic behaviour and on the spectroscopic properties of the carotenoid are presented.

INTRODUCTION

There is no information on the carotenoid composition of the photosynthetic tissues of the ten surviving genera of the Cycadales. In the literature [1], only a few data on seed coat carotenoids are given, and they indicate the presence of simple mixtures. The seed coat of *Cycas revoluta* contains zeaxanthin as the chief component (75%) with small amounts of cryptoxanthin and β-carotene (the so called "Type II mixture"). The seed coats of four Cycad genera [2], i.e. *Dion*, *Encephalartos*, *Macrozamia*, and *Zamia*, contain the "Type II mixture" and also the "Type I mixture" (lycopene as major component plus traces of β-carotene).

In our studies, we have concentrated upon the carotenoid composition of leaflets of *Ceratozamia fuscoviridis* D. Moore. *C. fuscoviridis* was described for the first time by D. Moore [3] as "a supposed new species of *Ceratozamia*". Much later on, it was described and identified by Schuster [4] as one of the forms of *C. mexicana* Brongn.

In our investigation, this form was examined because specimens were available from our collection at the Botanical Garden of Florence University. In the first stage of development of every new composite leaf, the leaflets (6–8 cm long) show a characteristic dark red-brown colour which progressively disappears during growth (*ca* 2 months). From a preliminary investigation [5] this transitory colour appeared as chiefly due to the occurrence of a keto-carotenoid which at the time of its largest presence can represent 70% of the total carotenoids of the leaflets. In addition we also investigated the carotenoid composition of a definately attributed species, *C. kuesteriana* Regel [6], the specimens of which came from the collection of the Botanical Garden of Naples University.

Our first chemical and spectroscopic results [5] have been partially revised and corrected, and the keto-carotenoid has been identified as the semi-β-carotenone in the specimens of both plants. For a long time this secocarotenoid was not known as a natural carotenoid but only as a product of the chemical oxidation of β-carotene. In 1968 it was isolated from a natural source, i.e. from the fruits of *Murraja exotica*, a citrus relative [7], but until now it has not been found in leaves, i.e. in a photosynthetic tissue.

In the leaves of *C. fuscoviridis* it is present in the chloroplasts but unlike the other carotenoids it is probably located not in the thylakoid membranes but in very electrondense bodies similar to the plastoglobuli which are found in the stroma.

RESULTS AND DISCUSSION

On TLC in the classic system [8], the compound isolated from young leaves of *C. fuscoviridis* and *C. kuesteriana* behaved as a weakly polar compound (R_f 0.68–0.73). This marked epiphasic character excluded the presence of a hydroxyl group and consequently a typical xanthophyll structure. Even after strong saponification the pigment maintained its epiphasic character and the same R_f, so that a xanthophyll ester structure was excluded. The epoxide test was negative. In addition, it ran with the same R_f if co-chromatographed with semi-β-carotenone obtained from the fruits of *M. exotica* [7].

The relationship between the molecular structure of the semi-β-carotenone from *C. fuscoviridis* and *C. kuesteriana* and its relative polarity (sum of the polarities of the individual functional groups of the molecule) was utilized as a complementary tool in its identification. The relative polarity values we used were the classic ones assigned by Krinsky [9] and were tabulated for 25 carotenoids of which only two keto-carotenoids each of which contained two conjugated carbonyl groups were considered. According to the classic rule, a conjugated carbonyl group contributes a relative polarity (rel. pol.) value of 0.72; if another conjugated carbonyl group is added to the molecule the keto contribution increases to 1.44 [9]. However, this rule did not consider the atypical case of semi-β-carotenone, at that time unknown, which is a monocyclic carotenoid with a conjugated and an unconjugated carbonyl group at the end of the open chain.

If the relationship between the relative polarity of semi-β-carotenone and its chromatographic behaviour on silica

gel is examined, the unconjugated carbonyl group, C(5')=O, present in the molecule, apparently does not contribute to the relative polarity of the molecule. In fact in the TLC system utilized for this type of test [10], semi-β-carotenone has a higher R_f value than either lutein (rel. pol. = 1.89) or lutein monoester (rel. pol. *ca* 1 or *ca* 0.89). In addition, it is more epiphasic than lutein monoester and has a rel. pol. (0.72) which is much lower than the expected value (1.44) if both carbonyl groups are involved.

The visible spectra, recorded in six organic solvents, showed a selective loss of fine structure. In fact, in the two apolar solvents we used, i.e. petrol (bp 40–60°) (λ_{max} nm: 466 and *ca* 485 sh) and particularly *n*-hexane (λ_{max} nm: *ca* 447 slight sh, 468 and *ca* 492 sh), the fine structure was still partially resolved. In benzene (λ_{max} nm: 482 and *ca* 502 slight sh) and carbon bisulphide (λ_{max} nm: 500 and *ca* 525 slight sh) only a slight shoulder other than the λ_{max} was shown. By contrast, in the two polar solvents, chloroform (λ_{max} nm: 480) and ethanol (λ_{max} nm: 473), the fine structure was completely lost. Finally, reduction of semi-β-carotenone, carried out in ethanol with sodium borohydride, gave the reduced product, which, after purification by TLC, absorbed in the same solvent at *ca* 425 (sh), 446 (λ_{max}) and 475 nm (hypsochromic shift of 28 nm) and at *ca* 420 (sh), 443 (λ_{max}) and 470 nm in *n*-hexane (hypsochromic shift of 25 nm).

The IR spectrum of semi-β-carotenone in carbon bisulphide showed a band at 1723 cm^{-1} assignable to the unconjugated carbonyl group at C-5', while the absorption of the conjugated carbonyl group at C-6' appeared at 1673 cm^{-1}, in accordance with the previously reported data [7].

The relative abundancies of the most significant peaks in the EI mass spectra of semi-β-carotenone from *C. fuscoviridis*, *C. kuesteriana* and *M. exotica* are given in the Experimental. It is known that $[M-92]^+$, loss of toluene, and $[M-106]^+$, loss of xylene, are typical and abundant in-chain fragments of any C_{40}-carotenoid. Recently, particular attention has been given to the abundance ratio of these two ions ($R = I_{M-92}/I_{M-106}$) [11]. For the most common bicyclic carotenoids, which usually contain nine conjugated double bonds, the $[M-92]^+$ ion has generally a much larger abundance than the $[M-106]^+$ ion. By contrast the monocyclic and acyclic carotenoids, which often contain more conjugated double bonds (10–13), show a marked prevalence of $[M-106]^+$ over $[M-92]^+$. In our case, the specific abundance ratio ($R = 0.15$) for the semi-β-carotenone from *C. fuscoviridis* and *C. kuesteriana* was in good agreement with the typical ranges of low values of R reported for many monocyclic and acyclic carotenoids [11].

The 70 eV mass data of semi-β-carotenone from *C. fuscoviridis* and *C. kuesteriana* were compared with those of an authentic sample of semi-β-carotenone obtained from fruits of *M. exotica* [7]. In each sample the molecular ion peak (*m/z* 568) was a neat singlet at a resolution of 12,000 (10% valley). The exact mass values found by the peak-matching technique were 568.425 and 568.424 for the samples from *C. fuscoviridis* and *M. exotica* respectively (calculated value: 568.428 for $C_{40}H_{56}O_2$).

Conclusive evidence for the identities of the samples was obtained by metastable ion (MI) analysis of the molecular ions generated by EI, with a typical MS/MS experiment [12], using the reversed geometry double focusing ZAB-2F instrument as a tandem mass spectrometer [13]. The mass-analysed ion kinetic energy (MIKE) MI spectra [14], which show the daughter ions originating from the preselected molecular precursors spontaneously dissociating in the second field-free region, are practically indistinguishable. Therefore dissociating molecular ions must have not only identical structures, but also identical internal energy distributions, which can be achieved only by ionizing the same neutrals under identical conditions. Interestingly the MIKE-MI spectrum (Fig. 1) shows a much greater abundance of $[M-92]^+$ ions than of $[M-106]^+$ ions, i.e. the reverse to that seen in the normal EI mass spectra.

A tedious and time consuming purification of the semi-β-carotenone from *C. fuscoviridis* was necessary in order to obtain enough material (*ca* 0.5 mg) for a high resolution ^1H NMR spectrum (see Experimental). The 300 MHz

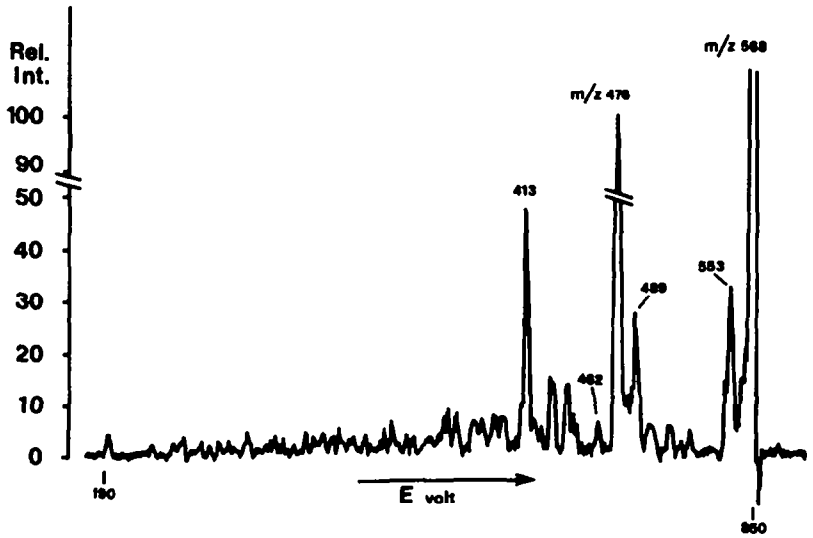

Fig. 1. Metastable ion (MI) mass-analysed ion kinetic energy (MIKE) spectrum of $[M]^+$ (*m/z* 568) of semi-β-carotenone from *C. fuscoviridis* upon electron ionization (EI) at 70 eV.

spectrum (probe temperature 25°) exhibited singlets at δ1.02 and 1.15 (*gem*-Me at C-1 and C-1', respectively), 1.70 (Me at C-5), 1.97 (Me at C-9, C-13, C-9' and C-13') other than the resonance of C-5' methyl group at δ2.1. The above data are in agreement with those reported in the literature [7, 15]. We could also detect strongly overlapped resonances at δ1.47, 1.51, 1.58 and at *ca* 1.62, the multiplicities of which were obscured by the presence of small amounts of water (δ1.54) in the sample but they could be reasonably assigned to the methylene groups at C-2, C-2', C-3 and C-3' [15]. The 4-CH_2 protons appeared as a shoulder at *ca* δ2 while the triplet at δ2.39 ($^1J_{3'\text{-H}}$ = 6 Hz) suggested the presence of the 4'-CH_2 protons deshielded by the neighbouring C(5') = O group [15]. The signals of the olefinic protons lie in the range from δ6.1 to 6.8 except for the 8'-H resonance which is found as a doublet centred at δ7.38 ($^1J_{7'\text{-H}}$ = 15 Hz) [15].

EXPERIMENTAL

Limbs of young and red-brown leaflets of *C. fuscoviridis* and of *C. kuesteriana* were homogenized in pure Me_2CO and, after complete extraction, the pigments of the crude Me_2CO extract were transferred to petrol by gently washing several times with distilled cold H_2O as in the classic transfer procedure. The dried ethereal epiphase was carefully concentrated to a small vol. under vacuum. The concentrated soln was subjected to semi-prep. TLC on silica gel (0.5 mm thickness), according to the classic procedure [8]. A sufficient aliquot of semi-β-carotenone was scraped off from several plates and, after elution with double distilled Me_2CO, chromatographed three or more times in the same system. The semi-purified material was then subjected to TLC on an inorganic adsorbent mixture according to another classic procedure [16]. Prior to TLC the plates were developed in pure Me_2CO in order to remove extraneous lipid impurities detectable under UV light. The greater part of the sterols and other similar impurities were removed the usual way, i.e. by cooling a concentrated *n*-hexane soln of the semi-β-carotenone at −20°.

As the last purification step, the semi-β-carotenone was subjected to HPLC on a 7 μm silica gel column (Violet, 25 × 0.4 cm) ($CHCl_3$–hexane, 1:4) with a flow rate of 1 ml/min and monitoring at 475 nm. This last step was repeated at least two times particularly for the 300 MHz ^{1}H NMR spectra. For this purpose the HPLC eluate was reduced in vol., adsorbed on a silica gel column (2 × 3.8 i.d. cm) and eluted with a few ml of hexane–Me_2CO (22:3).

All the pure grade solvents, particularly Me_2CO and *n*-hexane, were double distilled.

Every complementary tool of identification: that is epoxide test, control of eventual loss of the epiphasic character or increase of the polarity after saponification, co-chromatographic tests, comparison tests to elucidate the relationship between the molecular structure and the relative polarity [9] and other tests, previously and largely utilized [10], were conducted using silica gel TLC with the classic developing mixture [8].

The reduction of the carbonyl groups was performed with $NaBH_4$; the character of the reduced compound and the eventual reappearance of the fine structure were checked by the visible spectra.

The fruits of *M. exotica* were kindly supplied by the Botanical Garden of Palermo University, Sicily; from the skin of these fruits the semi-β-carotenone was extracted and purified by the procedures just described.

The MS on semi-β-carotenone from *C. fuscoviridis* and *M. exotica*, obtained by direct introduction of the samples into the ion source, kept at 200–220°, without heating the probe, gave the following values (*m/z*, in parenthesis the respective rel. int.): 568 (9 and 10), 476 (0.7 and 0.7), 462 (4.5 and 6), 368 (5 and 6), 81 (41 and 45), 69 (100 and 100), 55 (83 and 85), 43 (97 and 98).

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