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The chemical structure of the pigments in *Ara macao* plumage

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Abstract

Parrots (Psittaciformes) harbor unusually bright, non-carotenoid, feather pigments. We successfully extracted and purified a sufficient quantity of pigment from the red plumage of the Scarlet Macaw (*Ara macao)* for a partial chemical analysis. The extracts were analyzed by HPLC coupled with UV–VIS and mass spectroscopy before and after total hydrogenation. We found at least four pigment components. We propose a linear polyenal structure comparable with the molecules tetradecahexenal, hexadecaheptenal, octadecaoctenal and eicosanonenal. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Bird's plumage; Parrots; *Ara macao*; Color; Pigments; Biogenesis; Interaction with keratin

1. Introduction

With their stout hooked bill, thick prehensile tongue, zygodactyl feet, and great intellectual capacity, parrots are immediately distinguishable from any other group of birds. It has long been recognized that parrots harbor unusual bright-colored pigments in their feathers instead of carotenoids; already in 1882, Krukenberg recognized the unique character of these lipochromes, and gave them the name psittacofulvins (Krukenberg, 1882). The extracted pigments differed from the widely distributed carotenoids (then called zooerythrin and xanthoerythrin) in their apparent lack of absorption bands in the visible range, and in their chemical reactivities and solubilities.

Völker subsequently characterized these unusual pigments chemically and spectrally (Völker, 1936, 1937, 1942). When considering feathers of similar colours, the freed pigments from parrot feathers absorbed at much shorter wavelengths and had peaks of absorption closer together than the corresponding carotenoids (Völker, 1936). Unlike carotenoids, the pigments in parrots did not depend on dietary input of precursors (Völker, 1936). Finally, they are visible under ultraviolet light (Völker, 1936, 1937). To this day, the chemi-

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cal structures and biogenic affinities of the pigments in parrots have remained a mystery, which is made all the more compelling by the fact that only parrots produce these pigments endogenously.

Thanks to new developments in analytical chemistry, notably the development of tandem HPLC UV/VIS, it is finally possible to advance probable structures for these compounds.

Based upon a study of Raman resonance spectra 'in situ' on several Parrot species, an hypothesis was developed that the chromophase responsible for the coloration of the plumage of parrots was due to a linear polyene chain containing 7–9 conjugated double bonds (Veronelli et al., 1995). Recently, we have been able to extract and purify from the red plumage of *Ara macao*, a sufficient quantity of pigment for a partial structural analysis.

2. Materials and methods

2.1. Biological materials

Red feathers (10 g) of an individual of *Ara macao* (adult male) were a gift from Prof. Renato Massa, Milan University.

2.2. Chemicals and reagents

Methanol, acetonitrile and acetone were supplied by Merck (Darmstadt, Germany) and filtered through a membrane filter for small volumes of liquids, 13 mm diameter, 0.5 μm, from Millipore (Milford, MA, USA). $NaBH_4$, (4)–NO_2–C_6H_4–COCl, myristyl alcohol, cetyl alcohol, stearyl alcohol and eicosanol were supplied by Aldrich (Italy).

2.3. Extraction and purification procedure

Pigments were extracted and purified as follows.

Approximately 10 mg of colored barbules were carefully washed with hexane on a glass filter and finely ground in a micronizer Retsch MM2 (Germany) equipped with a ZrO container in the presence of 8 ml of methanol for one hour. The pigments were freed from keratin and the white residue (inorganic salts) filtered on Sep-Pak C_{18} Cartridges (Waters Millipore, Milford, MA, USA). The filtrate containing the pigments was evaporated under reduced pressure at room temperature. The residue was dissolved in 200 μl of acetone. After freezing for 3 h at −78°C, the supernatant was filtered. The above operations were repeated 30 times. The solutions were collected and evaporated under a stream of dry nitrogen. The residue was purified by column chromatography (SiO_2) eluting with hexane/ethyl acetate (90/10).

2.4. Apparatus and HPLC–UV/VIS analysis

A Gymcotec A 110 instrument equipped with an Isocratic Gymcotec Pump Mod. 300 (Munich, Germany) was employed for HPLC. Pigment analysis was carried out using two sequential Lichrocart Purosphere RP-18 Columns (250 × 4 mm I.D.) maintained at 40°C by column block heater (model 7970, Hichrom Ltd., Reading, UK). The solvents were degassed with helium before use. The mobile phase was acetonitrile/methanol (70/30) and the flowrate was 0.5 ml min^{-1}. Samples of the pigments and their derivatives were injected with a Rheodyne 7125 Valve equipped with 20-μl loop. Data were acquired between 230 and 600 nm with a Diode Array Detector, HP 1050 series, using a HP Chem Software, and three-dimensional chromatograms were recorded.

The HPLC procedure is the same as that employed for the analysis of carotenoid patterns in other bird plumage (Stradi et al., 1995).

2.4.1. HPLC–MS analysis

Mass spectra were recorded with a HP 5988A particle beam instrument equipped with a HP 1050 HPLC. The same column used for HPLC analysis was employed. Samples were ionized by negative chemical ionization (CI) with methane. The ionization energy was 240 eV.

2.5. Chemical transformations of pigment

2.5.1. Reduction with $NaBH_4$

The crude extract (∼10 mg) was dissolved in dry ethanol (1 ml) and treated under stirring with 4 mg of $NaBH_4$. After 12 h, the solvent was evaporated under vacuum and the residue dissolved in the HPLC mobile phase and injected for HPLC analysis.

2.5.2. Reduction with H_2/PtO_2 and derivatization with (4)–NO_2–C_6H_4–COCl

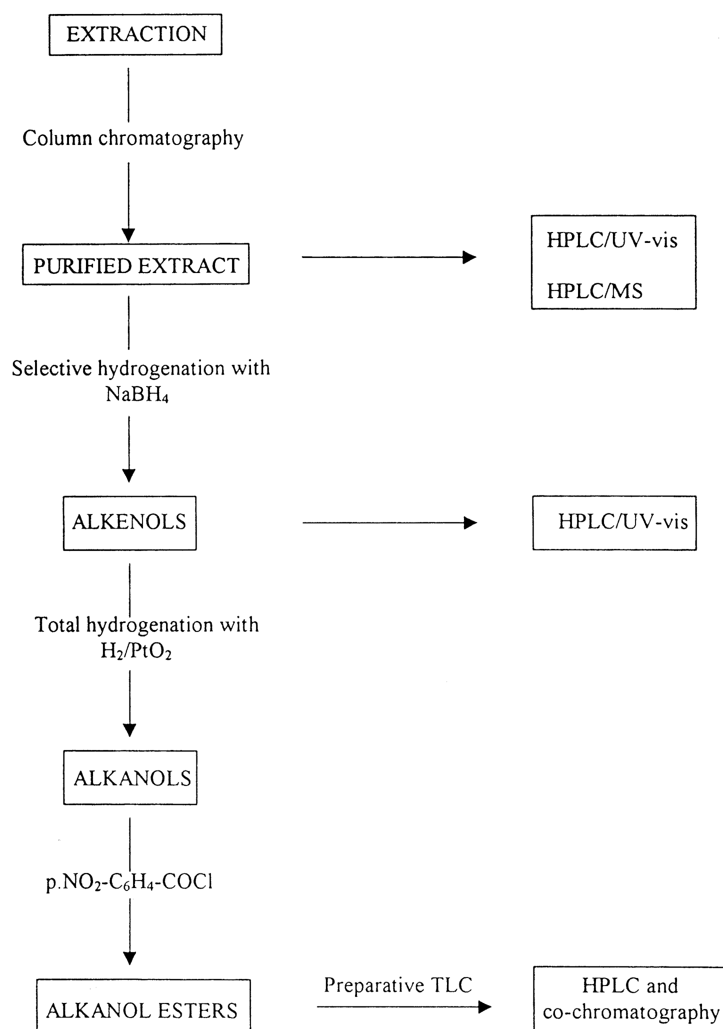
To the crude extract (10 mg) dissolved in methanol (2 ml), PtO_2 (approx. 2 mg) and hydrogen were added at room temperature and pressure until the yellow color disappeared. The solution was filtered and solvent evaporated under vacuum; the residue was dissolved in $CHCl_3$ (3 ml) and (4)–NO_2–benzoylchloride (5 mg), and triethylamine (2 drops) were added. After standing (4 h), the solvent was evaporated under reduced pressure and the crude residue was washed (three times) with an aqueous saturated solution of $NaHCO_3$, and extracted with ethyl acetate (2 × 5 ml). The organic layer was concentrated to 500 μl and the (4)–NO_2–benzoylesters developed by preparative TLC on Merck silica gel plates (20 × 20 cm) with ethyl acetate/hexane (20/80).

Pure myristyl, cetyl, stearyl and eicosanoyl esters were prepared as described above and employed as chromatographic standard without further purification.

3. Results

Extraction, purification and analysis of the pigment were performed following Scheme 1.

In the experimental conditions employed, the HPLC UV/VIS and mass analysis of the pigments showed four main peaks with a R.T. range between 5.5 and 7.5 min. Electronic spectra ap-



Scheme 1. Isolation, purification and analysis of plumage lipochromes.

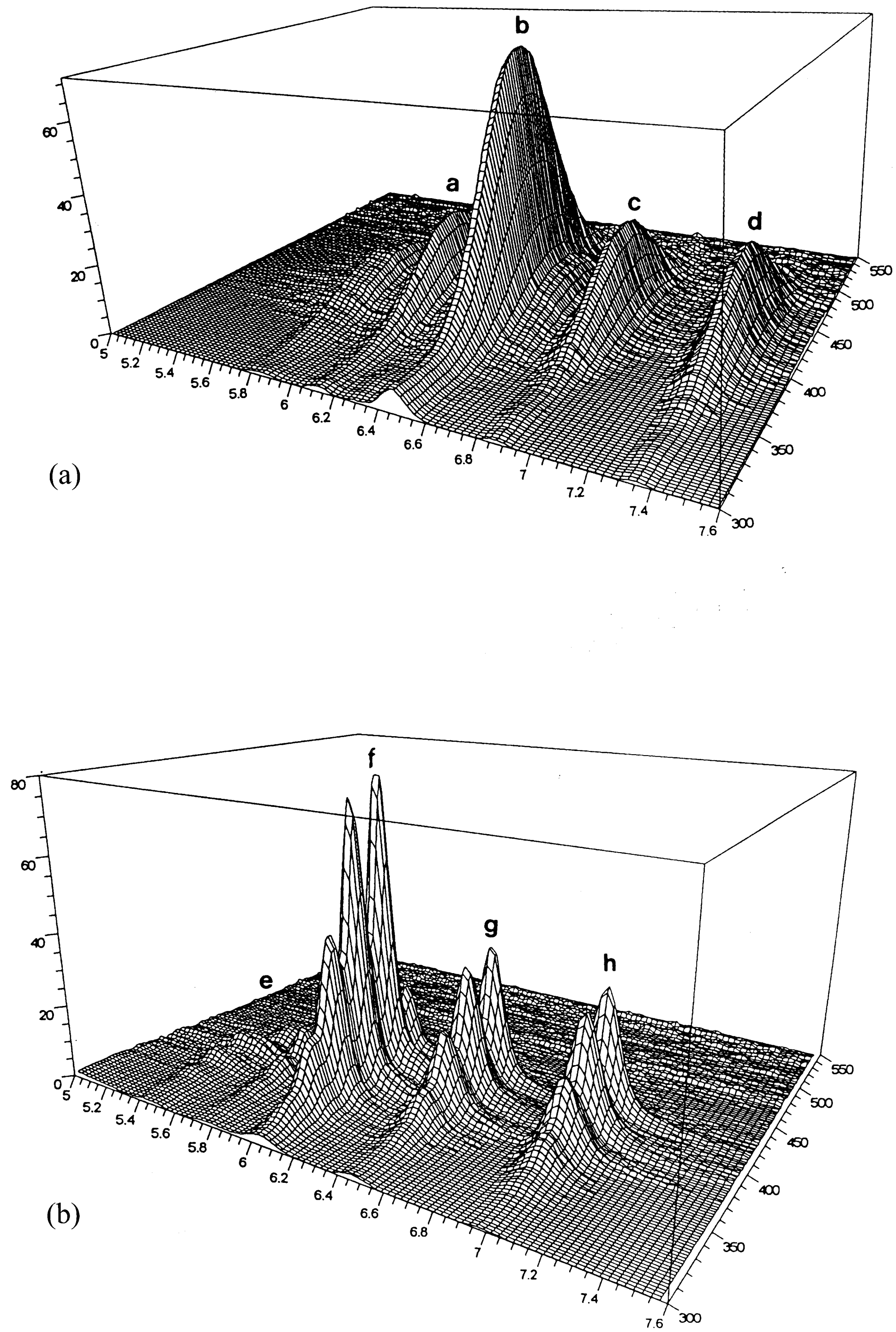


Fig. 1. Three-dimensional chromatograms of (a) extracted pigment and (b) after $NaBH_4$ reduction.

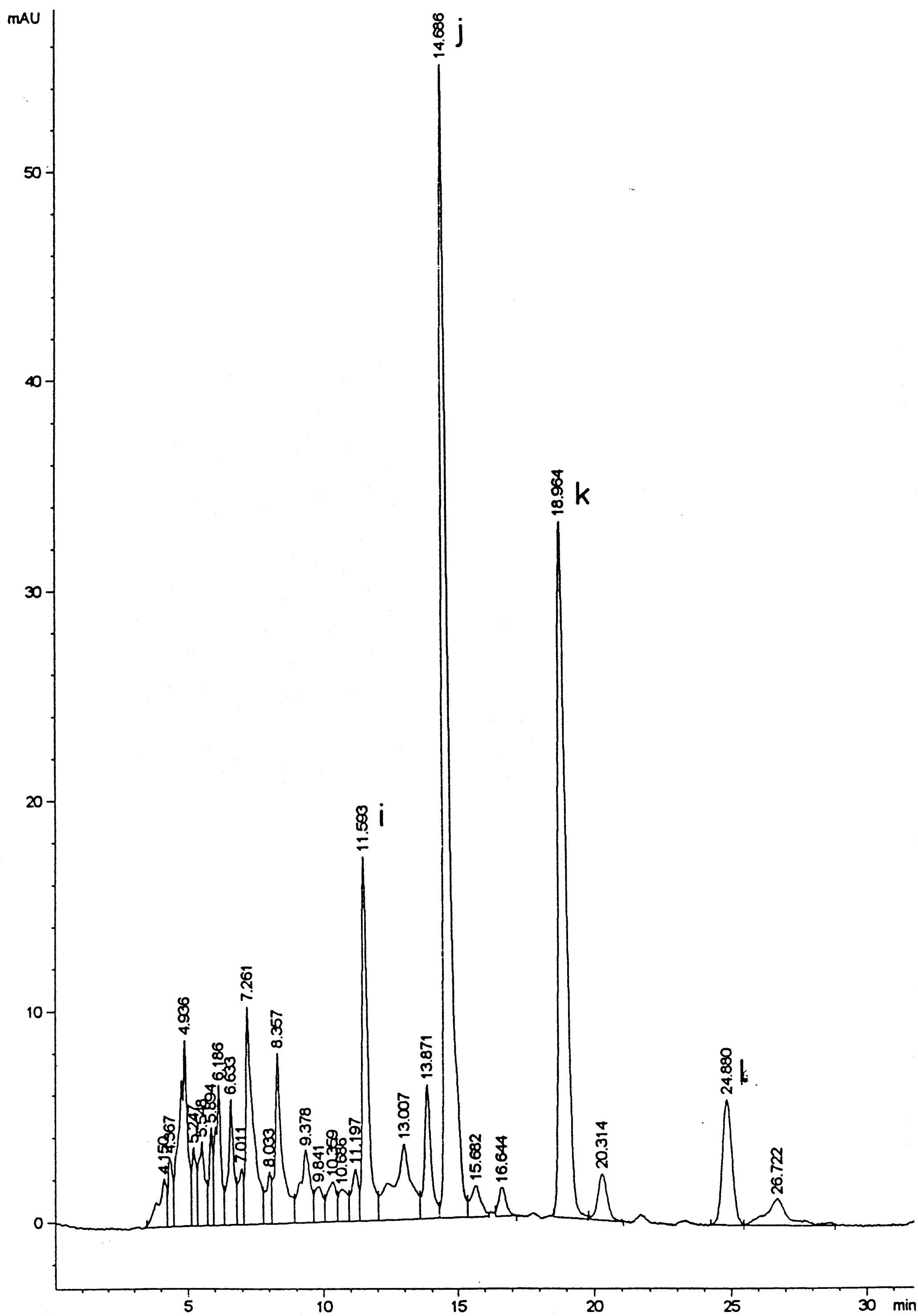


Fig. 2. HPLC analysis of esterification products of totally hydrogenated pigment, detected at λ = 254 nm.

pear without vibronic structure and the λ_{max} is between 400 and 450 nm (Fig. 1a). Their molecular masses are 200, 226, 252 and 278 Da, respectively.

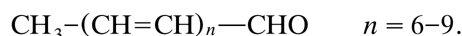
The pigments reduced with $NaBH_4$ show a similar chromatographic pattern for absolute and relative retention times, but differ dramatically in their electronic spectra, which shift to a lower λ_{max} and show a neat vibronic structure (Fig. 1b).

Total hydrogenation (H_2/PtO_2) of the pigments as such, or after reduction with $NaBH_4$, afforded a mixture of myristyl alcohol, cetyl alcohol, stearyl alcohol, and eicosanol in which cetyl alcohol represents the main component.

These four alcohols were identified by HPLC as (4)–NO_2–benzoyl esters. Their identification was confirmed by co-chromatography with authentic samples (Fig. 2).

Table 1 shows HPLC and UV/VIS parameters of the pigments and of the corresponding $NaBH_4$-reduced products.

HPLC chromatography, coupled with UV/VIS spectroscopy and mass spectrometry of the extracted pigment and hydrogenated (selectively or totally) derivatives, permits us to formulate the hypothesis that the pigments responsible for the color consist of a mixture of four polyenals with general formula A:



$$CH_3\text{–}(CH{=}CH)_n\text{—}CHO \qquad n = 6\text{–}9.$$

The structures of the minor (tetradecahexenal, a, $n = 6$) and the main components (hexadecaheptenal, b, $n = 7$) were confirmed by comparison (HPLC, UV/VIS) with authentic samples prepared following the Kuhn procedure (Kuhn and Grundmann, 1937; Blout and Fields, 1948). The same applies to their $NaBH_4$ reduction products, e and f.

The structures of pigment components with higher retention times were tentatively assigned from their molecular weight, HPLC, UV/VIS properties and those of the products derived from their $NaBH_4$ reduction (e and f) as octadecaoctenal (c, $n = 8$) and eicosanonenal (d, $n = 9$).

These structural assignments are strongly supported by the results of total hydrogenation of the pigment, which produces a mixture of saturated alcohols C_{14} (i), C_{16} (j), C_{18} (k) and C_{20} (l).

In conclusion, polyenals A were reduced with $NaBH_4$ to polyenols which upon hydrogenations using H_2/PtO_2, gave the corresponding alkanols:

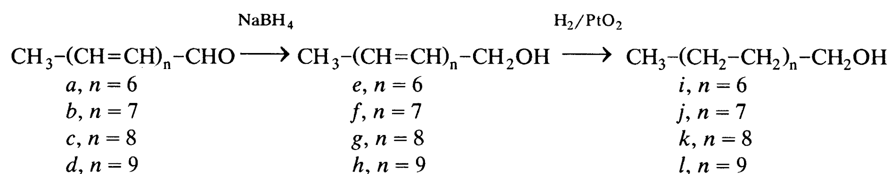


Table 1
HPLC UV/VIS parameters of pigments and of the corresponding $NaBH_4$ reduced products

Compound	RT (min)	λ_{max} (nm)[a]
a	6.10	403
b	6.45	420
c	6.85	436
d	7.35	445
e	5.75	335,351,370
f	6.05	362,373,395
g	6.45	375,395,418
h	7.00	385,417,430

[a] Solvent: acetonitrile/methanol (70/30).

4. Discussion

The linear polyenals responsible for the feather coloration would be expected to be synthesized from a chain of C_2 (acetate) units. Two known biosynthetic pathways could do this.

4.1. The polyketide pathway

From acetyl CoA, a chain of acetate units is formed, which after enzymatic reduction followed by dehydration, would produce the polyenic chain (Fig. 3). This pathway is well known in plants, fungi and bacteria, but we are unaware of any examples in animals.

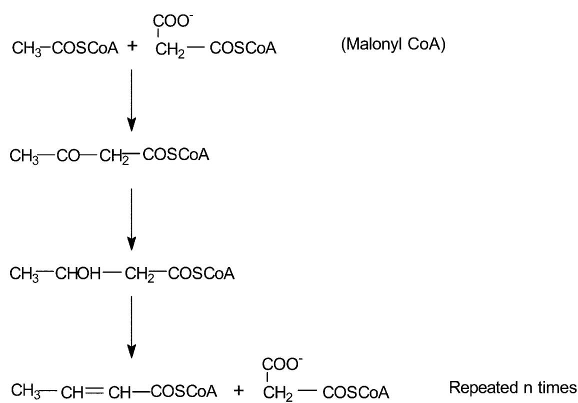


Fig. 3 Polyketide pathway of acetyl CoA

4.2. A modified fatty acid pathway: C_2 units are added stepwise (Fig. 4)

It is also possible that this particular group of birds have enzymes that can desaturate fatty acids (see Fig. 4).

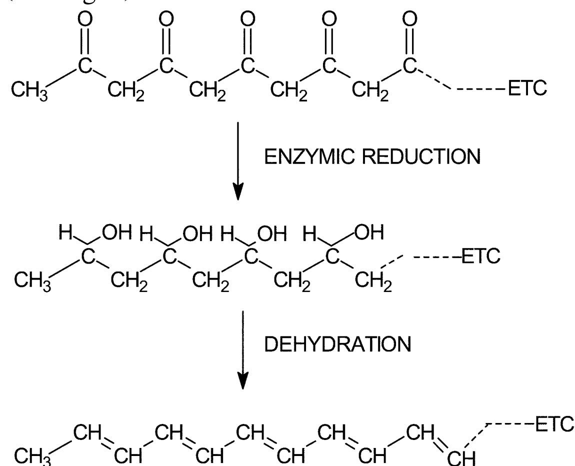


Fig. 4 Modified fatty acid pathway

This is a fascinating result, which suggests novel biochemical phenomena, which need to be tested experimentally.

Our next goal is to investigate the type of interaction between the polyenals and the plumage keratin. This interaction is responsible for the dramatic red-shift from the isolated pigment to the keratin-linked pigment. This bathochromic effect could be of supramolecular nature, as observed in some yellow carotenoids linked to the red feathers of *Carduelis carduelis*, or due to covalent bonds as in the case of retinol linked to opsin in rhodopsin (Del Zoppo et al., 1998).

We expect to demonstrate that the brilliant colors of the parrot plumage are principally due to such interactions, and that parrots construct their rainbow of color simply by modulating the interaction of a few endogenous yellow pigments with the plumage keratin.

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