Bioorganic Chemistry 162 (2025) 108623

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Modifying the chromophoric ring of betalamic acid in plant pigments. Synthesis and characterization of methylated betalains☆

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ARTICLE INFO

Keywords:
α-Methyl-DOPA
Betalamic ring
Betalain chemistry
Methylated pigment

ABSTRACT

The recent discovery of decarboxylated betalains, derived from dopamine, in nature has expanded the number of chemical modifications possible in these plant pigments. Alternative starting materials in biochemical processes can yield novel molecules with modifications in the betalamic ring. For the first time, a functional group has been incorporated into the betalamic acid structure of betalains, thus creating a novel family of methylated pigments. A synthetic pathway starting from α-methyl-DOPA yields 6-methyl-betalamic acid. The process is enzymatic and kinetic characterization reveals that the formation of 6-methyl-betalamic acid is favored over the formation of 6-methyl-dopaxanthin, as a further condensation reaction can be hindered. Starting from the methylated betalamic acid, a novel family of methylated betalains has been biotechnologically produced comprising ten yellow and violet molecules. The novel compounds were characterized by using HPLC-ESI-Q-TOF-MS, spectrophotometry, and spectrofluorometry. Potential antioxidant capacity was measured using the ORAC assay, demonstrating the molecules' effectiveness against peroxyl radicals, with indoline-6-methyl-betacyanin identified as the most antioxidant. This study shows the venue for the chemical modification of the betalamic ring in pigments and thus represents the beginning of the era of betalain core chemistry. This opens new fields for future research to explore novel structures and their potential bioactive effects.

1. Introduction

In plants of the order Caryophyllales, betalains are nitrogenous, water-soluble compounds that serve as the main pigments [1,2], with the exception of the families Caryophyllaceae and Molluginaceae, where anthocyanins are responsible for coloration [3]. Betalains contain betalamic acid [4-(2-oxoethylidene)-1,2,3,4-tetrahydropyridine-2,6-dicarboxylic acid] as their chromophoric and structural unit, with betaxanthins formed when this molecule condenses to amino acids and amines, and betacyanins resulting from its condensation with indoline-type molecules such as *cyclo*-dihydroxyphenylalanine (*cyclo*-DOPA) or its glycoside derivatives [4]. These molecules exhibit characteristic coloration: yellow-orange for betaxanthins, with a maximum absorbance around 480 nm, and red-violet for betacyanins, with a maximum absorbance close to 536 nm [5]. This coloration arises from the extension of the system of conjugated double bonds present in betalamic acid. In addition, betaxanthins display a strong fluorescence [6]. The biosynthetic pathway of betalains begins naturally in plants with the hydroxylation of L-tyrosine to produce 3,4-dihydroxy-L-phenylalanine (L-DOPA) by enzymes cytochrome P450 [7]. Subsequently, the enzyme 4,5-DOPA-extradiol-dioxygenase (4,5-DODA) uses L-DOPA as substrate and catalyzes the cleavage of the aromatic ring to form the

☆ This article is part of a Special issue entitled: 'Green and sustainable' published in Bioorganic Chemistry.

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https://doi.org/10.1016/j.bioorg.2025.108623
Received 18 March 2025; Received in revised form 21 May 2025; Accepted 21 May 2025
Available online 23 May 2025

intermediate compound 4,5-seco-DOPA [8]. This molecule undergoes spontaneous intramolecular condensation, yielding betalamic acid.

A new family of natural betalains has recently been described, characterized by a decarboxylated betalamic acid (6-decarboxy-betalamic acid) able to condense with amines and amino acids to form decarboxylated betaxanthins and betacyanins [9]. In this case, the enzyme 4,5-DODA utilizes dopamine as an alternative substrate and, similarly to the traditional pathway, generates the intermediate 4,5-seco-dopamine, ultimately producing 6-decarboxy-betalamic acid [10]. Considering that the biosynthetic pathways of the two betalain families known to date originate from different but structurally related substrates, the use of alternative molecules that may lead to the formation of novel betalains is explored. In this work, for the first time, the substrate promiscuity of the enzyme 4,5-DODA is exploited to synthesize methylated betalains from the substrate α-methyl-DOPA. In the proposed new biosynthetic pathway, the action of the enzyme 4,5-DODA on α-methyl-DOPA may result in the intermediate molecule 4,5-seco-methyl-DOPA, which is ultimately converted into 6-methyl-betalamic acid, a potential structural unit of methylated betalains. Consequently, it is possible to obtain betalains with a methyl group in their structural unit (Fig. 1). Until now, efforts to produce novel betalains have focused on modifying the condensable portion of the molecule that interacts with betalamic acid. Here, a completely new approach to betalain chemistry is introduced, involving the production of new pigments through modifications directly in the structure of the betalamic ring. Unlike decarboxylated betalains, where the natural pigments lack a carboxyl group, the newly described family of methylated betalains incorporates a methyl group into the betalamic ring. This represents the first addition of a functional group to the chromophoric and structural core of betalains. Accordingly, the targeted synthesis of these methylated molecules has been initiated by using α-methyl-DOPA as a unique precursor not previously explored in pigment formation. This study describes the production and characterization of 6-methyl-betalamic acid through the activity of the enzyme 4,5-DODA on α-methyl-DOPA. In contrast to L-DOPA and dopamine, both present in nature, α-methyl-DOPA has an artificial origin [11], and the methylated intermediates derived from it and the new family of methylated betalains are considered purely synthetic compounds. Here, the production and purification of ten methylated betalains, members of a novel class of pigments, is described, being the pigments characterized from the molecular, spectroscopic, and antioxidant perspectives. The described findings broaden the understanding of how the use of an alternative precursor may allow

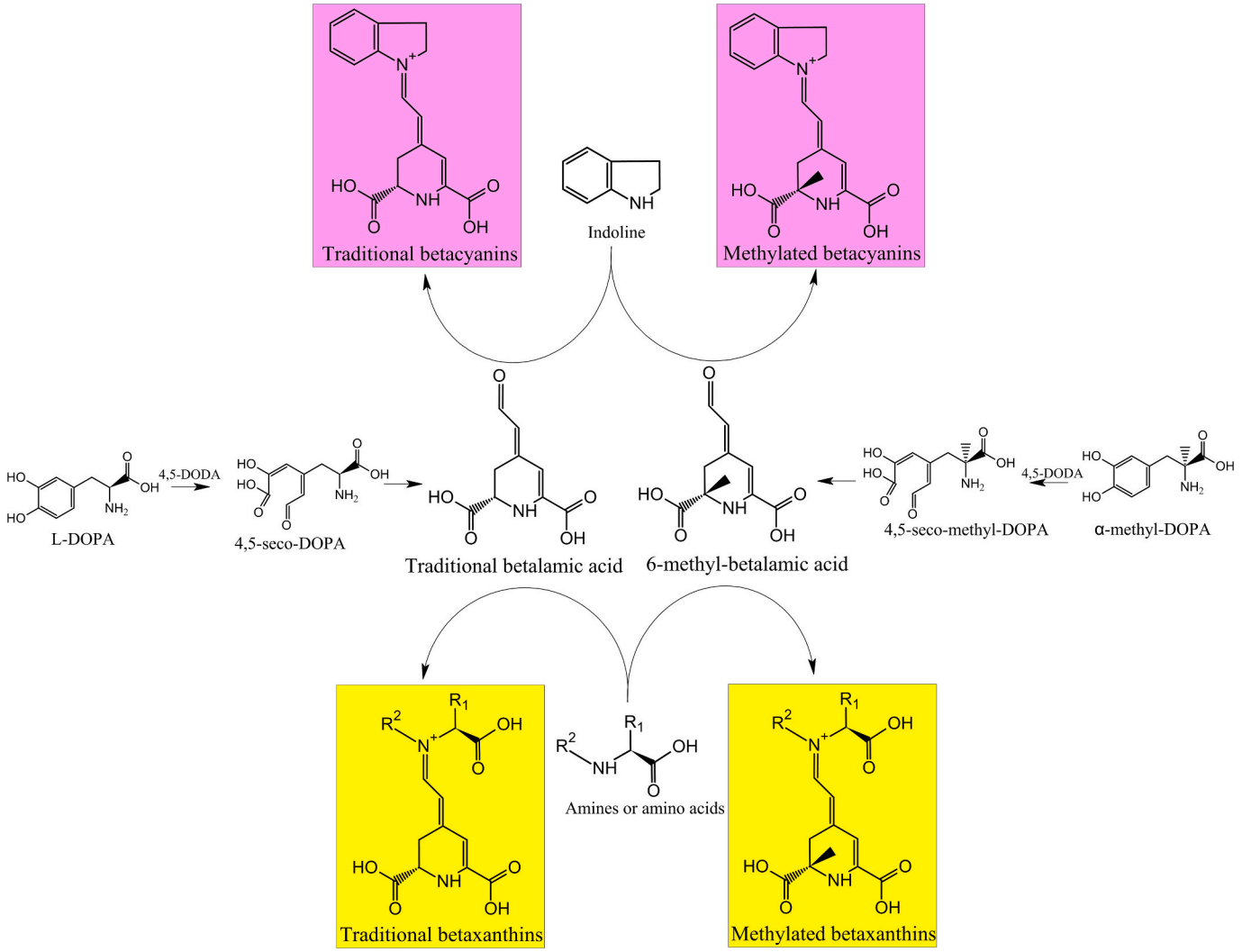

Fig. 1. Representative scheme illustrating the synthesis pathway of traditional and methylated betalains from the substrates L-DOPA and α-methyl-DOPA, respectively, using 4,5-extradiol-dioxygenase (4,5-DODA) enzyme. Transformations leading to the formation of yellow pigments (betaxanthins) and violet pigments (betacyanins) via condensation reactions between betalamic and 6-methyl-betalamic acid with amines/amino acids or indoline-type molecules, respectively, are shown. DOPA, dihydroxyphenylalanine. R_1 and R_2 indicate H or the side chain of the amine group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the biosynthesis of a unique family of betalains with direct modifications to the betalamic ring, which influence their properties and bioactivity. This work opens new avenues to produce structural variations of the molecules of this family, as well as for the investigation of their properties and bioactive effects, which could lead to industrial, food or pharmaceutical applications.

2. Experimental section

2.1. Chemical reagents

Sodium ascorbate, isopropyl β-D-1-thiogalactopyranoside (IPTG), kanamycin (Km), chloramphenicol (Cn), trifluoroacetic acid (TFA), sodium hydroxide (NaOH), hydrochloric acid (HCl), Luria-Bertani (LB) culture medium, sodium acetate ($NaC_2H_3O_2$), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and fluorescein were obtained from Sigma-Aldrich (St. Louis, MO, USA). The following compounds were also obtained from Sigma-Aldrich: (−)-3-(3,4-dihydroxyphenyl)-2-methyl-L-alanine sesquihydrate (α-methyl-DOPA), pyrrolidine-2-carboxylic acid (L-proline), (2*S*)-2-amino-4-methylsulfinylbutanoic acid (L-methionine sulfoxide), 9-tetrahydropyrrole (pyrrolidine), (2*S*)-2-amino-3-(1H-indol-3-yl)propanoic acid (L-tryptophan), 2-phenylethylamine (L-phenylethylamine), (2*S*)-2-amino-3-phenylpropanoic acid (L-phenylalanine), (2*S*,4*R*)-4-hydroxyproline-2-carboxylic acid (L-hydroxyproline), 2-amino-3-(1H-imidazol-5-yl)propanoic acid (L-histidine), (*S*)-(−)-indoline-2-carboxylic acid (indoline-COOH), and indoline. Water and acetonitrile were purchased from Fisher Scientific (Dublin, Ireland).

2.2. Strains and plasmids

The enzyme-mediated synthesis of 6-methyl-betalamic acid was done by expressing the enzyme 4,5-DODA from the proteobacterium *Gluconacetobacter diazotrophicus* (GdDODA, WP_012222467.1) in the bacterium *Escherichia coli* Rosetta 2 (DE3) [12]. The pET28 expression vector was used for transforming the bacteria.

2.3. Kinetic characterization of the enzyme 4,5 DODA on the substrate α-methyl-DOPA

The enzyme 4,5-DODA was obtained and purified following the protocol published by Contreras-Llano and coauthors (2019) [12]. A continuous spectrophotometric approach was used to measure the absorbance obtained at $\lambda = 414$ nm in order to assess the enzyme activity on the substrate α-methyl-DOPA [13,14]. Reaction products were analyzed by HPLC following the protocol published by Gandía-Herrero and coauthors (2005) [15].

Activity measurements were conducted at different pH values using 50 mM sodium acetate-HCl buffers (pH 3.5–5.5) and 50 mM sodium phosphate buffers (pH 5.5–8.5). The total reaction volume was 300 μL. Reactions consisted of 50 mM of the selected buffer, 6.5 mM α-methyl-DOPA, and 10 mM sodium ascorbate. Measurements were carried out at 25 °C in 96-well plates using a Synergy HT plate reader (Bio-Tek Instruments, Winooski, USA). The plate reader's signal detector was calibrated using known concentration betalamic acid solutions. For the quantification of α-methyl-betalamic acid, the reported molar extinction coefficient at 424 nm ($\varepsilon = 24{,}000\ M^{-1}\ cm^{-1}$) for betalamic acid was used as reference value [16]. Additionally, a Jasco V-650 spectrophotometer (Easton, MD, USA) was used to monitor the enzymatic reaction's progress in the ultraviolet and visible spectrum. Repetitive spectra were recorded every 10 min over 190 min, scanning wavelengths from 250 to 700 nm. Experiments were conducted at 25 °C in 1 mL quartz cuvettes. All assays were performed in triplicate. Kinetic analysis of the data was performed using nonlinear regression fitting with SigmaPlot software (Version 14, Systat Software Inc., Palo Alto, CA, USA).

2.4. Synthesis of methylated betalains

Ten structurally related betalains were synthesized from 6-methyl-betalamic acid. The pigments were produced after condensation with amines and amino acids following the protocol previously described [17,18]. Briefly, bioreactors of *E. coli* cells harboring the previously described expression vector for the aromatic ring cleaving enzyme were prepared with LB medium at a concentration of 20 g L^{-1} and supplemented with the antibiotics Km (100 mg mL^{-1}) and Cn (34 mg mL^{-1}). Cultures were incubated with orbital shaking at 37 °C until reaching an optical density (OD) at 600 nm between 0.8 and 1. The expression of 4,5-DODA was induced by the addition of IPTG (1 mM), followed by overnight incubation at 20 °C. Subsequently, the culture was centrifuged at 5000 rpm and 4 °C for 10 min to discard the supernatant (LB). The pellet was resuspended in sterile water and transferred to 50 mL centrifuge tubes, then centrifuged at 7500 rpm and 4 °C for 10 min. Afterward, the supernatant was removed, and the pellet was resuspended in sterile water supplemented with sodium ascorbate (15 mM) and α-methyl-DOPA (1 mM). In addition, amines/amino acids (10 mM) were added for the formation of different methylated betaxanthins; indoline (0.38 mM) and indoline-COOH (3.8 mM) were added for the formation of methylated betacyanins. Bioreactors were maintained at 20 °C with shaking for 72 h. Finally, cultures were centrifuged at 7500 rpm and 4 °C for 10 min, and the supernatant was stored at −20 °C until the purification stage.

2.5. Purification of methylated betalains by reversed-phase chromatography

Novel methylated betalains were purified by using preparative HPLC. Before purification, samples were filtered through a 0.2 μm pore size, 25 mm diameter filter. The purification process was carried out using a Shimadzu Nexera Prep HPLC system (Kyoto, Japan), equipped with an LC-20AP pump and an SPD-M40 PDA photodetector. The procedure involved reversed-phase chromatography using a Kinetex 5 μL/C-18 column (250 × 21.2 mm, Phenomenex, Torrance, CA, USA). Following protocols described in the literature [15,18], water and acetonitrile, both supplemented with 0.05 % (v/v) TFA, were used as solvents. The acetonitrile gradient ranged from 0 % to 35 % over 25 min, with a flow rate of 25 mL min^{-1} and an injection volume of 5 mL. Fractions containing the desired molecule were collected and pooled. TFA present in the samples was neutralized with NaOH to reach a pH of 5.5–6.0. Then, the acetonitrile present in the purified samples was removed using rotary evaporation. The aqueous solutions for each pure individual methylated betalain were stored at −20 °C until further analysis. Since they are water-soluble pigments, the characterization of betalains by HPLC-ESI-Q-TOF-MS, spectrophotometry and spectrofluorimetry is performed using the compounds in aqueous solution. The compounds were not obtained in an isolated powder form due to their low yield, thus their long-term storage stability remains unresolved.

2.6. HPLC analysis

Methylated betalains diluted in water were analyzed using an analytical Shimadzu LC-10 A HPLC system (Kyoto, Japan) equipped with an SPD-M10A photodiode array detector. The analysis was conducted by reversed-phase chromatography using a Kinetex C-18 column (250 × 4.6 mm, 5 μm particle size) (Phenomenex). The chromatographic method involved a linear gradient over 25 min, from 0 % to 35 % of solvent B, with a flow rate of 1 mL min^{-1} and an injection volume of 50 μL at 30 °C. Solvents A and B were water (0.05 % TFA) and acetonitrile (0.05 % TFA), respectively [18].

2.7. Analysis by mass spectrometry

The structural confirmation of the new methylated betalains was performed by mass spectrometry using an Agilent 6550 Q-TOF-MS spectrometer equipped with a dual electrospray ionization interface (Agilent JetStream Dual ESI) and following the protocol and conditions described in previous studies [9]. The chromatographic method used was the same as that described for the HPLC analyses (section 2.6). Each detected peak yielded an experimental mass (*m/z*), which was used to calculate its deviation, Δppm, relative to the theoretical mass of the tentative molecule [9]. The determination of Δppm values served as a method for confirming elemental composition, with values between −5 and 5 ppm being accepted for the identification of different compounds [19,20].

2.8. Spectrophotometric and spectrofluorometric characterization

Molar extinction coefficient (ε) of 6-methyl-betalamic acid was obtained using the synthesized compound tryptophan-6-methyl-betaxanthin. Hydrolysis of this novel compound with ammonia produces 6-methyl-betalamic acid and L-tryptophan, the concentration of which can be determined by HPLC [16] using a calibration curve for the amino acid L-tryptophan. The concentration of L-tryptophan released during hydrolysis is equal to the initial concentration of tryptophan-6-methyl-betaxanthin and to the 6-methyl-betalamic in the hydrolysis medium. Following the protocol described in the literature [18], the ε value for 6-methyl-betalamic acid was calculated by applying the 1:1 ratio in the hydrolysis of the betaxanthin and Lambert-Beer's law using the absorbance value at 414 nm due to methylated betalamic acid.

Then, the absorption spectrum for each methylated betalain was obtained using the spectrophotometer described in section 2.3 with scans from 350 to 700 nm, using a 1 mL quartz cuvette. The ε value for each methylated betalain was determined using the same ammonia degradation method [18]. After obtaining the spectra for each methylated betalain, diluted ammonia (1:10 in water) was added to the cuvette for pigment degradation. Hydrolysis was monitored for 30 min, with cumulative spectra recorded every 2 min. The concentration of the resulting 6-methyl-betalamic acid was then determined using its ε, which is equal to the initial concentration of the betalain (1:1 stoichiometry). Once the concentration for each betalain and the initial absorbance values of the solutions are known, the ε values were calculated by applying Lambert-Beer's law [18].

The spectrofluorometric characterization of the methylated betalains was performed using a Shimadzu RF-6000 spectrofluorometer (Kyoto, Japan). Excitation spectra were obtained by recording emission at the λ_{max} of emission, and emission spectra were recorded by exciting each betalain at its corresponding λ_{max} of excitation. The pigment concentration used for the measurements was 3 μM.

2.9. ORAC assay

The antioxidant activity of the methylated betalains was studied using the oxygen radical absorbance capacity (ORAC) assay. This method is based on the ability to protect the fluorescent molecule fluorescein from peroxyl radicals generated by 2,2′-azobis(2-methyl-propionamidine) dihydrochloride (AAPH). For this purpose, a reaction mixture (200 μL) was prepared with fluorescein (37.5 nM), AAPH (19 mM), and different concentrations of methylated betalains (2.5, 1.25, 0.5, and 0.05 μM) dissolved in 75 mM sodium phosphate buffer, pH 7.4. Trolox was used as a reference antioxidant. The measurements were performed in 96-well plates using the reader described in section 2.3. The experiments were conducted in triplicate. Data analysis was performed using linear regression fitting with SigmaPlot.

3. Results and discussion

3.1. Oxidative cleavage of α-methyl-DOPA by the DODA enzyme: Kinetic characterization

The activity of the recombinant *G. diazotrophicus* DODA enzyme in the presence of a reaction medium with α-methyl-DOPA was spectrophotometrically characterized. The addition of the enzyme caused the appearance of a yellow coloration, with a $\lambda_{max} = 414$ nm (Fig. 2A). The spectral change was not observed in the absence of the recombinant protein, and thus, it is considered a result of a novel activity detected for the *G. diazotrophicus* enzyme. The described activity aligns with previously performed spectrophotometric observations of the absorbance of betalamic acid and muscaflavin extracted from plants and fungi [16,21,22], and of the same compounds obtained in vitro by the dioxygenases from *Amanita muscaria, Mirabilis jalapa, B. vulgaris,* and *E. coli* [8,13,14,23]. A pH variation was performed in the reaction medium, resulting that the optimal pH for the activity of the *G. diazotrophicus* enzyme in the transformation of α-methyl-DOPA was 6.5 (Fig. 2B).

Kinetic characterization of the transformation of α-methyl-DOPA by the DODA enzyme showed a reduction in the activity at high concentrations of α-methyl-DOPA (Fig. 2C, Supplementary Fig. S1). This phenomenon hinders the production of the novel metabolite, and it is known as inhibition by excess of substrate of the responsible enzyme. Methyl-DOPA acts as a substrate able to block the active site of the enzyme when present in high concentrations, thus reducing the transformation rate. This effect corresponds to a specific kinetic model, where the Lineweaver–Burk plot or double reciprocal plot of the activity rate (inverse activity rate versus the inverse concentration of α-methyl-DOPA) provides a typical V-shaped curve, as shown in Fig. 2D [24]. The equation for the obtained curve yielded the kinetic parameters Km = 88 M and $V_{max} = 56.8$ mol min^{-1}, with a substrate inhibition constant as low as 8.86×10^{-5} mM. The highest rate (0.91 mmol min^{-1}) was obtained at a concentration of 2.5 mM α-methyl-DOPA, which is the optimal concentration for this enzyme-catalyzed reaction. The Km obtained is higher than anticipated for a kinetic model without inhibition, since this rate is impacted by the significant substrate inhibition mentioned, making it not comparable to the maximum rate in the Michaelis-Menten model [25]. Previous studies reported for GdDODA using dopamine as the substrate a strong substrate inhibition, with a Km = 18 M [10]. However, a Km value for GdDODA of 1.4 mM was described for the substrate L-DOPA [12] with no apparent inhibition. Despite the substrate inhibition, the DODA enzyme catalyzed the cleavage of the aromatic ring of α-methyl-DOPA across a wide range of concentrations and pHs and thus was a valued catalyst in the production of novel methylated compounds.

3.2. Oxidative cleavage of α-methyl-DOPA by the DODA enzyme: Intermediates of reactions

The transformation of α-methyl-DOPA was evaluated by HPLC. A medium containing the aromatic amino acid and purified GdDODA was allowed to react, and it was then analyzed after 3 h by reversed-phase chromatography HPLC. The analysis revealed complex medium with a total of 4 well-defined peaks in the chromatograms at wavelengths λ = 360, 405, and 480 nm (Fig. 3A, B, and C, respectively), which were not present in the absence of the enzyme. These peaks showed a distribution in the chromatograms similar to those observed in the analysis of reaction mixtures after the oxidative cleavage of L-DOPA [12] and dopamine [10]. Both compounds experience intramolecular cyclization after the oxidative cleavage of the aromatic ring due to the presence of the amine substituent at the appropriate distance to generate a nucleophilic addition with a subsequent proton transfer [12]. This rearrangement is also possible for the compound α-methyl-DOPA. Considering the peak distribution in the chromatograms of the reaction mixtures using L-DOPA and dopamine, it was proposed for methyl-DOPA that the peak

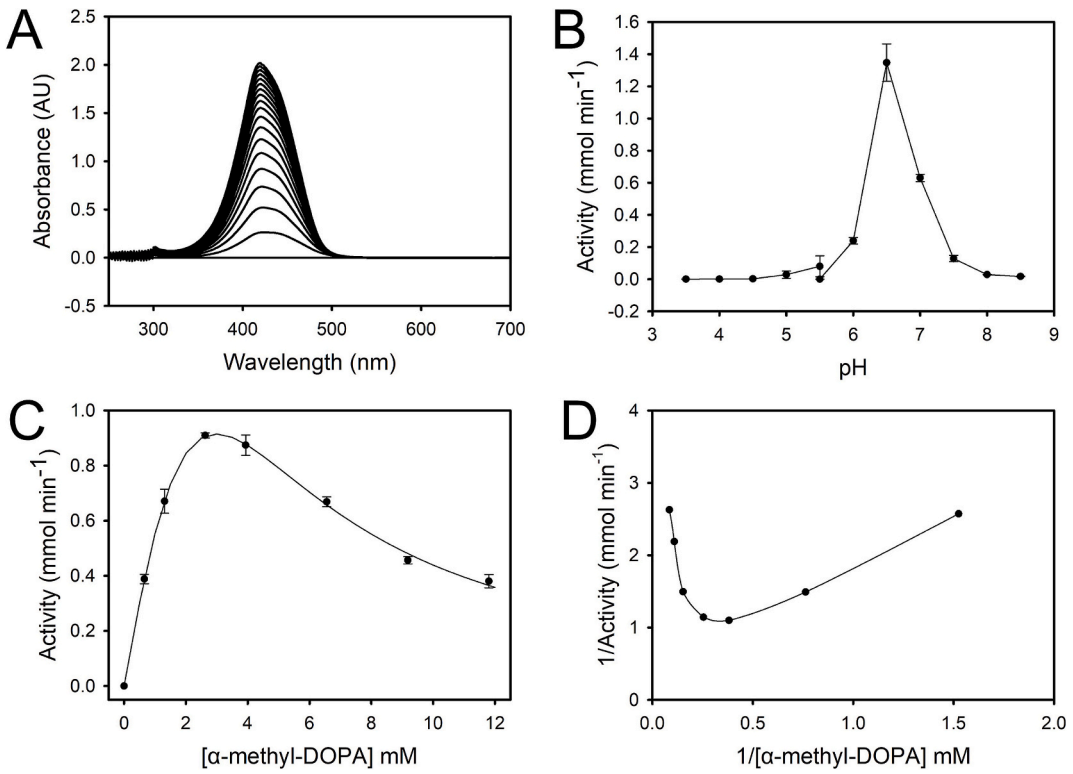

Fig. 2. Transformation of α-methyl-DOPA by the dioxygenase activity (DODA) of *Gluconacetobacter diazotrophicus*. (A) Spectral evolution of the transformation of α-methyl-DOPA (6.5 mM) by adding pure GdDODA enzyme to a reaction medium that contained sodium phosphate buffer 50 mM, pH 6.5, and sodium ascorbate 10 mM. (B) Effect of pH on the transformation of methyl-DOPA. Reactions were performed with 6.5 mM α-methyl-DOPA in 50 mM sodium acetate buffer (from pH 3.5 to 5.0) and in 50 mM sodium phosphate buffer (from pH 5.5 to 8.5). (C) Cleaving activity measured at different concentrations of α-methyl-DOPA, measured in 50 mM sodium phosphate buffer pH 6.5. (D) The inverse activity rate as a function of the inverse substrate concentration is displayed in a double-reciprocal plot. Every experiment was carried out in triplicate.

with a retention time (RT) of 18.04 min (Fig. 3B, Peak 3) and $\lambda_{max} = 404$ nm corresponded to 6-methyl-betalamic acid, while the peak with an RT of 19.74 min (Fig. 3B, Peak 4) and $\lambda_{max} = 401$ nm corresponded to 6-methyl-muscaflavin. The peaks with RTs of 10.20 and 12.87 min (Fig. 3A, Peaks 1 and 2) showing $\lambda_{max} = 361$ nm and $\lambda_{max} = 390$ nm, respectively, would correspond to 2,3- and 4,5-seco-methyl-DOPA, respectively. The higher RT of the peaks derived from α-methyl-DOPA compared to the peaks of compounds in reaction mixtures using L-DOPA [12] and dopamine [10] could be due to the presence of the methyl group, which increases their hydrophobicity. It is also noteworthy that, in comparison with reaction mixtures obtained for L-DOPA and dopamine, the reaction mixture with α-methyl-DOPA shows much lower production of the 2,3-DOPA-extradiol dioxygenase isomer (6-methyl-muscaflavin), suggesting that the methyl group in this molecule could hinder the nucleophilic attack of the amine group to this specific position, and thus the formation of 6-methyl-muscaflavin. Furthermore, it is also noteworthy the absence of an expected peak corresponding to pigment in the chromatogram at $\lambda = 480$ nm, compared to the analysis of reaction mixtures of L-DOPA [12] and dopamine [10]. This peak would correspond to the betaxanthin that should form from the condensation of 6-methyl-betalamic acid and the α-methyl-DOPA available in the medium. Since using L-DOPA as substrate rapidly leads to a betaxanthin derived from its condensation with traditional betalamic acid (dopaxanthin), the methyl group in α-methyl-DOPA could be hindering the reaction between the amine group of α-methyl-DOPA and the aldehyde group of 6-methyl-betalamic acid towards the formation of imines (Schiff condensation), thus avoiding the formation of the expected betaxanthin. In Fig. 3D, the proposed synthetic pathway to produce all the compounds detected is illustrated.

3.3. Oxidative cleavage of α-methyl-DOPA by the DODA enzyme: Time evolution of reaction products

Chemical intermediates were stable enough to allow HPLC analyses to follow the evolution of 4,5-methyl-seco-DOPA and 2,3-methyl-seco-DOPA which cyclization leads to 6-methyl-betalamic acid and 6-methyl-muscaflavin respectively. All these compounds have been identified for the first time. The enzyme-catalyzed and spontaneous reactions were monitored by HPLC for up to 40 h to characterize the formation and evolution of the intermediates and the accumulation of the final products 6-methyl-betalamic acid and 6-methyl-muscaflavin.

Supplementary Fig. S2 shows the evolution of intermediates 2,3- and 4,5-methyl-seco-DOPA measured at 360 nm (peaks 1 and 2, respectively). Both molecules appear immediately after the addition of the catalyst. 4,5-methyl-seco-DOPA reaches its maximum concentration 174 min after the reaction was triggered, while 2,3-methyl-seco-DOPA reaches its maximum presence after 462 min. Afterward, the amount of both molecules begins to decrease (Supplementary Fig. S3). In the case of 4,5-methyl-seco-DOPA, a rapid exponential decrease takes the concentration to almost zero. The evolution of the peak corresponding to 2,3-methyl-seco-DOPA is slower and follows a more linear pattern, without reaching the total disappearance of the compound. In the same manner, the evolution of 6-methyl-betalamic acid and 6-methyl-muscaflavin is shown in Supplementary Fig. S4 (peaks 3 and 4, respectively) and Supplementary Fig. S5. In both cases, a lag period is observed at the beginning of the experiment, and accumulation leads to plateaus towards the end (Supplementary Fig. S5). 6-methyl-betalamic acid reaches this plateau of maximum concentration at 1614 min, while 6-methyl-muscaflavin takes longer and reaches its maximum at the end of the experiment. In the case of 6-methyl-betalamic acid, the plateau is not completely stable and a slight decrease in concentration is observed.

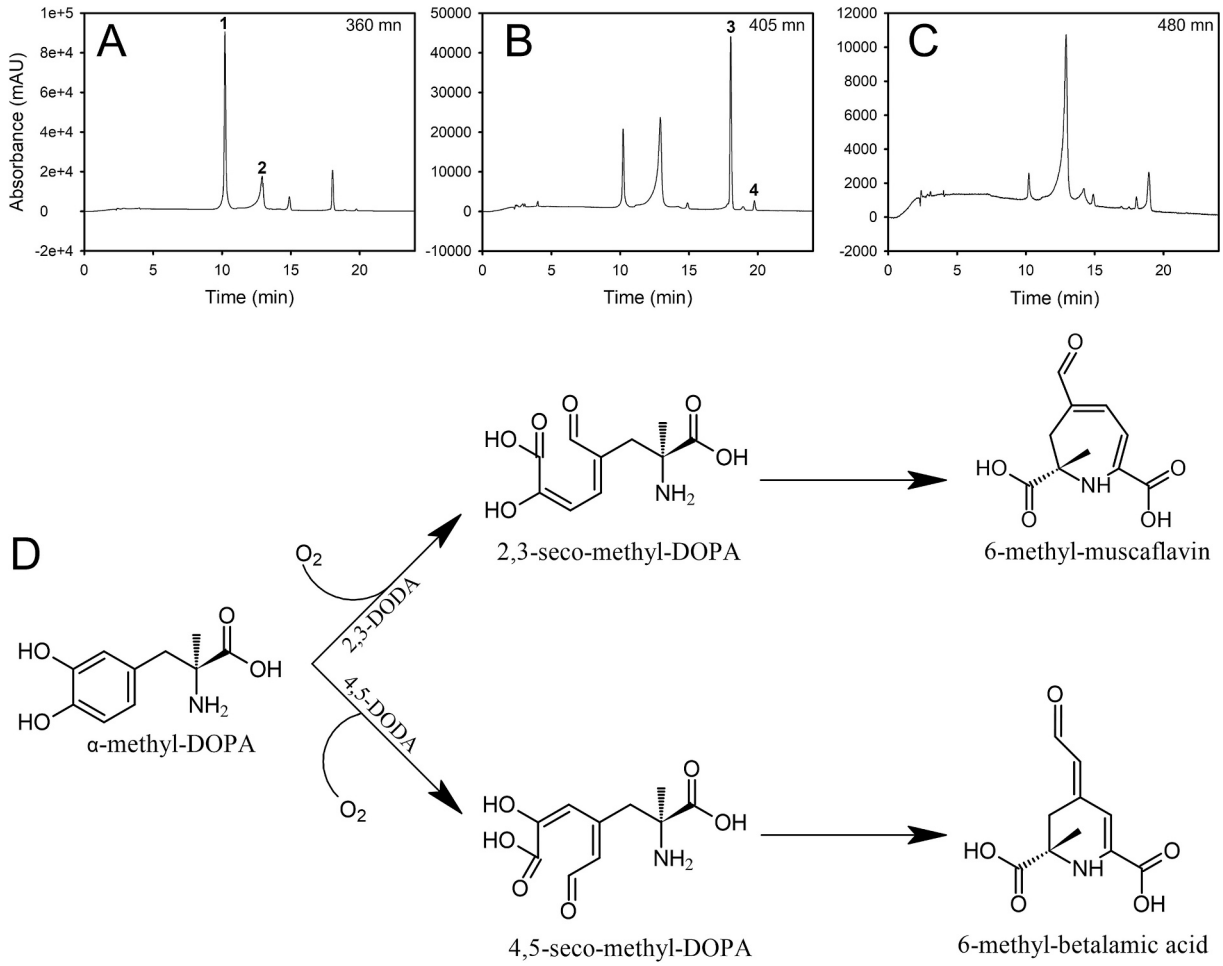

Fig. 3. HPLC analysis of reaction products formed from α-methyl-DOPA by oxidative cleavage of the aromatic ring and further intramolecular cyclization. Chromatograms for a reaction mixture comprising 6.5 mM α-methyl-DOPA and 10 mM sodium ascorbate in phosphate buffer (50 mM, pH 6.5) at 25 °C 3 h after the addition of pure GdDODA enzyme were obtained at λ = 360 nm (A), λ = 405 nm (B), and λ = 480 nm (C). Molecules shown are tentatively identified as 2,3-methyl-seco-DOPA (peak 1), 4,5-methyl-seco-DOPA (peak 2), 6-methyl-betalamic acid (peak 3), and 6-methyl-muscaflavin (peak 4). (D) Enzymatic-chemical mechanism for the formation of 2,3-methyl-seco-DOPA, 4,5-methyl-seco-DOPA, 6-methyl-muscaflavin and 6-methyl-betalamic acid from α-methyl-DOPA.

This may be due to the formation of a product from the condensation of 6-methyl-betalamic acid and α-methyl-DOPA molecules (Supplementary Fig. S6). As commented above, this reaction may not be favored since starting molecules lacking the methyl group tend to form betaxanthins to a much higher extent [10,12]. Analogously, the formation of the product derived from the 2,3-extradiol cleavage of the aromatic ring is much lower than that described for previously characterized substrates L-DOPA and dopamine [10,12], which only differ in the absence of the methyl group attached to the alpha carbon of the molecule. This, combined with the slower disappearance rate of the 2,3-intermediate compared to the 4,5-intermediate, suggests that the methyl group could hinder, to some degree, the cyclization of this intermediate to the 6-methyl-muscaflavin molecule. After normalizing the data obtained, the time evolution of the four novel molecules described is shown in Fig. 4. This representation clearly shows the relation between intermediates and the final products.

3.4. *Oxidative cleavage of α-methyl-DOPA by the DODA enzyme: Characterization of reaction products by mass spectrometry*

The reaction products derived from the 2,3 and 4,5-extradiol oxidative cleavage of α-methyl-DOPA were characterized by ESI-MS. The mass value determined for peaks 1 and 2 shown in Supplementary Fig. S2 was 244.2 *m/z* in both cases, corresponding to 2,3-seco-methyl-DOPA and 4,5-seco-methyl-DOPA, respectively. This is the expected value for the protonated molecular ions $[M+H]^+$ of both isomeric forms. Furthermore, both isomers exhibited similar fragmentation patterns, with major daughter ions at 154.0 *m/z* and secondary daughter ions at 108.2 and 226.0 *m/z*. In this same analysis, the mass values for peaks 3 and 4 (as shown in Supplementary Fig. S4), tentatively identified as 6-methyl-betalamic acid and 6-methyl-muscaflavin, respectively, were determined. The mass value for both of them was 226.1 *m/z*, consistent with the expected mass values for the protonated molecular ions $[M+H]^+$ of both isomers. In addition to this result, both molecules showed an identical fragmentation pattern, with a main daughter ion at 180.0 *m/z*, corresponding to the loss of a carboxyl group (−46 *m/z*). A similar phenomenon has previously been reported as characteristic of compounds with a similar structure [23]. Although traces of a betaxanthin were tentatively identified at longer reaction times (Supplementary Fig. S6, Peak 5), its low concentration did not allow detection by ESI-MS analysis.

The exact mass of the protonated molecular ions for the compounds 4,5-seco-methyl-DOPA, 2,3-seco-methyl-DOPA, 6-methyl-betalamic acid, and 6-methyl-muscaflavin was also determined by Time-of-Flight Mass Spectrometry (TOF-MS). The protonated molecular ions $[M+H]^+$ were found with an exact experimental mass of 244.0826 *m/z* for 4,5-seco-methyl-DOPA and 2,3-seco-methyl-DOPA; and 226.0727 *m/z* and 226.0709 *m/z* for 6-methyl-betalamic acid and 6-methyl-muscaflavin, respectively. These masses align with the calculated theoretical masses for 4,5-seco-methyl-DOPA and 2,3-seco-methyl-DOPA (244.0816 *m/z*),

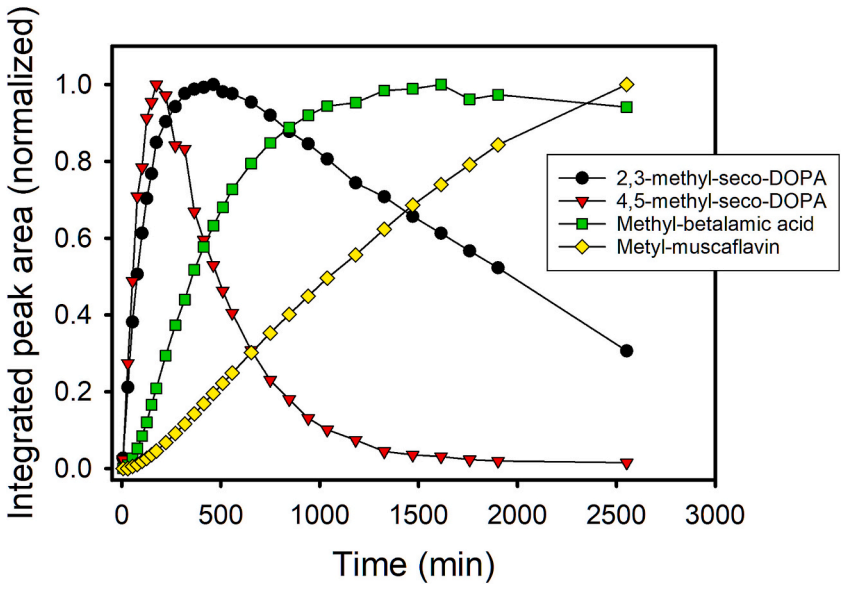

Fig. 4. Evolution of intermediates and final products in the enzymatic–chemical process that produces methylated betalamic derivatives. Normalized data are based on compound content determination by HPLC at the wavelengths 360 nm (seco-methyl-DOPAs) and 405 nm (6-methyl-betalamic acid and 6-methyl-muscaflavin). The reaction medium contained sodium phosphate buffer 50 mM, pH 6.5, α-methyl-DOPA 6.5 mM, and sodium ascorbate 10 mM. Measurements were taken at 25 °C.

with a difference of 4.09 ppm, and 6-methyl-betalamic acid and 6-methyl-muscaflavin (226.0710 *m/z*), with differences of 7.51 and −0.44 ppm, respectively. These analyses further support the structural determination of the intermediates and final products by yielding molecular formulas for the analyzed compounds as follows: $C_{10}H_{14}NO_6$ (4,5-seco-methyl-DOPA and 2,3-seco-methyl-DOPA, Peaks 1 and 2 in Supplementary Fig. S2) and $C_{10}H_{12}NO_5$ (6-methyl-betalamic acid and 6-methyl-muscaflavin, Peaks 3 and 4 in Supplementary Fig. S4). This analysis confirms the involvement of the intermediates 4,5-seco-methyl-DOPA and 2,3-seco-methyl-DOPA in the formation of 6-methyl-betalamic acid and 6-methyl-muscaflavin, respectively. The data obtained from the mass spectrometry analyses are summarized in Table 1.

3.5. Synthesis and structural characterization of methylated betalains

It has been demonstrated that the condensation of 6-methyl-betalamic acid with the α-methyl-DOPA molecule occurs very slowly, probably due to the presence of the methyl group. Since this group in the 6-methyl-betalamic acid moiety is not close to the carbonyl group involved in the imine formation, it is possible that the condensation reaction with other amines or amino acids might be possible to produce 6-methyl-betalains (Fig. 5). The resulting molecules have not been described to date, thus representing a novel and uncharacterized family of pigments.

The novel molecules were produced by adding amine-containing molecules capable of condensing with the 6-methyl-betalamic acid formed after the oxidative cleavage of α-methyl-DOPA. For this purpose, bioreactors were prepared with *E. coli* bacteria harboring the gene for the GdDODA able to yield 6-methyl-betalamic acid. 72 h after the reaction was started by adding the substrates, the bioreactors presented yellow/orange or pink/violet coloration, indicating the production of betaxanthins or betacyanins, respectively, depending on the amine added. Once obtained, purification of each novel compound was carried out by preparative HPLC. Subsequent analysis by analytical HPLC showed the existence of a single peak, corresponding to a pigment and confirming the purity of the compounds, tentatively considered methylated betalains (Fig. 6). We propose that the chemical nomenclature of this family of molecules should be based on the methylation of the new structural unit: 6-methyl-betalamic acid. Consequently, the pigments are named as 6-methyl-betalain pigments or methylated betalains. The stock concentration, yield, and chromatographic purity percentage obtained by HPLC for each compound are shown in Supplementary Table S1.

As also occurred for 6-methyl-betalamic acid, in comparison to traditional betalains [17,26], the new methylated pigments elute between 0.56 and 1.75 min later in reverse-phase chromatography analytical HPLC. A similar shift of 0.8–2.20 min was described for decarboxylated betalains, a family of betalainic pigments present in plants of the family Amaranthaceae [9,10,18] (Table 2). The shift was expected and justified by the fact that the methylation of betalains makes the structures more hydrophobic.

As seen in Supplementary Fig. S7, three-dimensional chromatograms were used to verify the level of purity of each pigment. In every case, a single compound was detected in the UV–visible range (350–800 nm).

Structural confirmation of each novel pigment was performed by HPLC-ESI-Q-TOF-MS, and the exact molecular mass and chemical formula of each pigment synthesized from α-methyl-DOPA were precisely determined. Table 3 shows the experimental mass values (*m/z*) and their

Table 1
Mass spectrometry data for intermediates and final products derived from the oxidative cleavage of α-methyl-DOPA.

Compound	Molecular Formula	$[M+H]^+$ (*m/z*)	Main-daughter ion (*m/z*)	TOF exact mass		
				Theoretical (*m/z*)	Experimental (*m/z*)	Δppm
2,3-seco-methyl-DOPA	$C_{10}H_{14}NO_6$	244.2	154	244.0816	244.0826	4.09699051
4,5-seco-methyl-DOPA	$C_{10}H_{14}NO_6$	244.2	154	244.0816	244.0826	4.09699051
6-methyl-betalamic acid	$C_{10}H_{12}NO_5$	226.1	180	226.0710	226.0727	7.51976149
6-methyl-muscaflavin	$C_{10}H_{12}NO_5$	226.1	180	226.0710	226.0709	−0.44233891

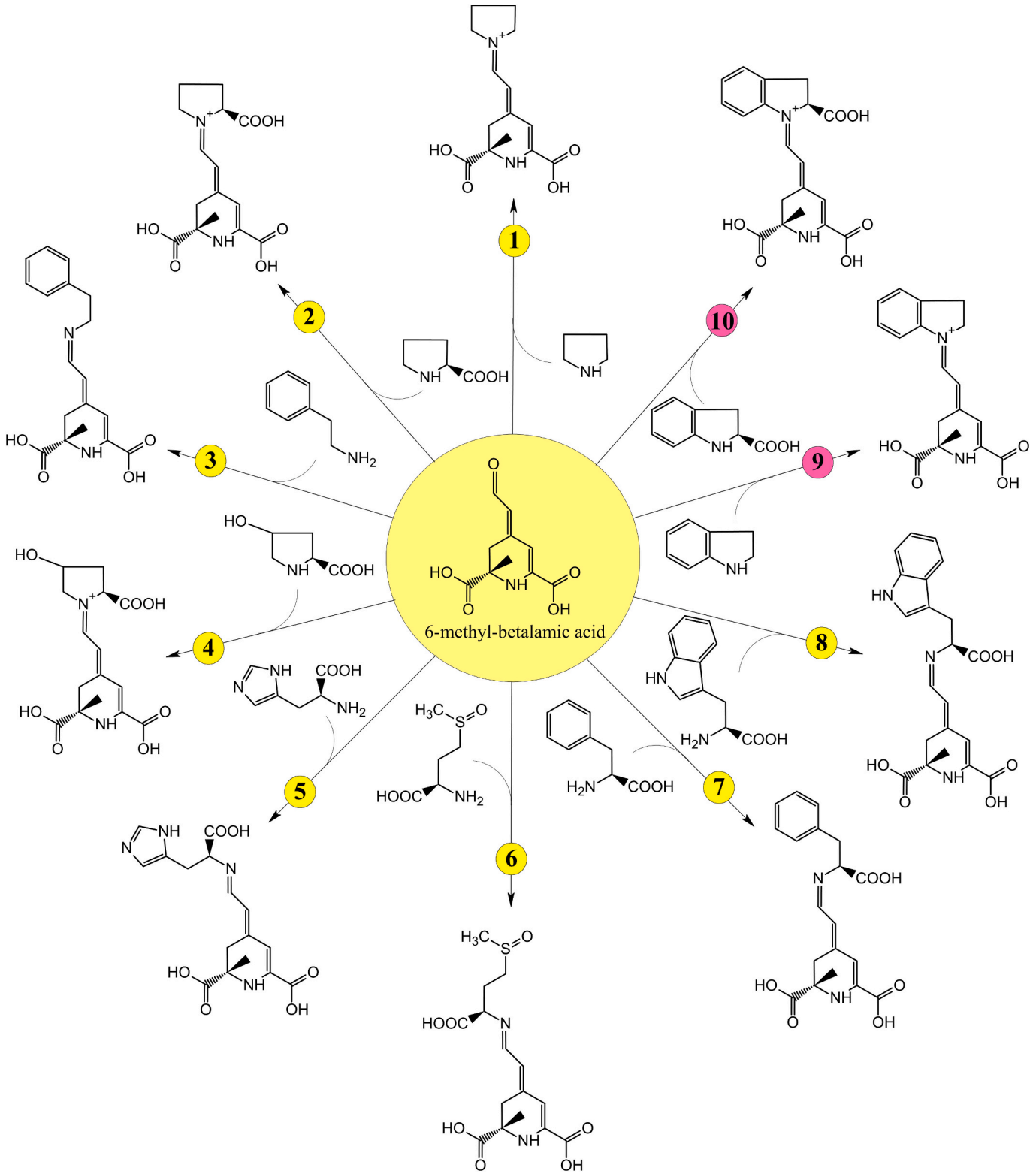

Fig. 5. Scheme representing the reaction between 6-methyl-betalamic acid and (1) pyrrolidine, (2) proline, (3) phenylethylamine, (4) hydroxyproline, (5) histidine, (6) methionine sulfoxide, (7) phenylalanine, (8) tryptophan, (9) indoline, and (10) indoline COOH to yield methylated betalains.

difference, in Δppm, with respect to the theoretical masses calculated. In addition, the acquired fragmentation data showed that all pigments lost at least one carboxyl group (Supplementary Fig. S8A), which agrees with previous research on betalains fragmentation [9,12]. Also, the isotopic distribution profile revealed a strong relationship between the mass spectra of each new molecule and the suggested formula (Supplementary Fig. S8B). In agreement with previously established valid Δppm ranges [19,20], the data obtained unambiguously identify the ten novel compounds as previously unconsidered methylated betalain pigments. Thus, it has been shown that the use of α-methyl-DOPA as an alternative substrate for the DODA enzyme results in the formation of methylated betalamic acid, which ultimately yields methylated

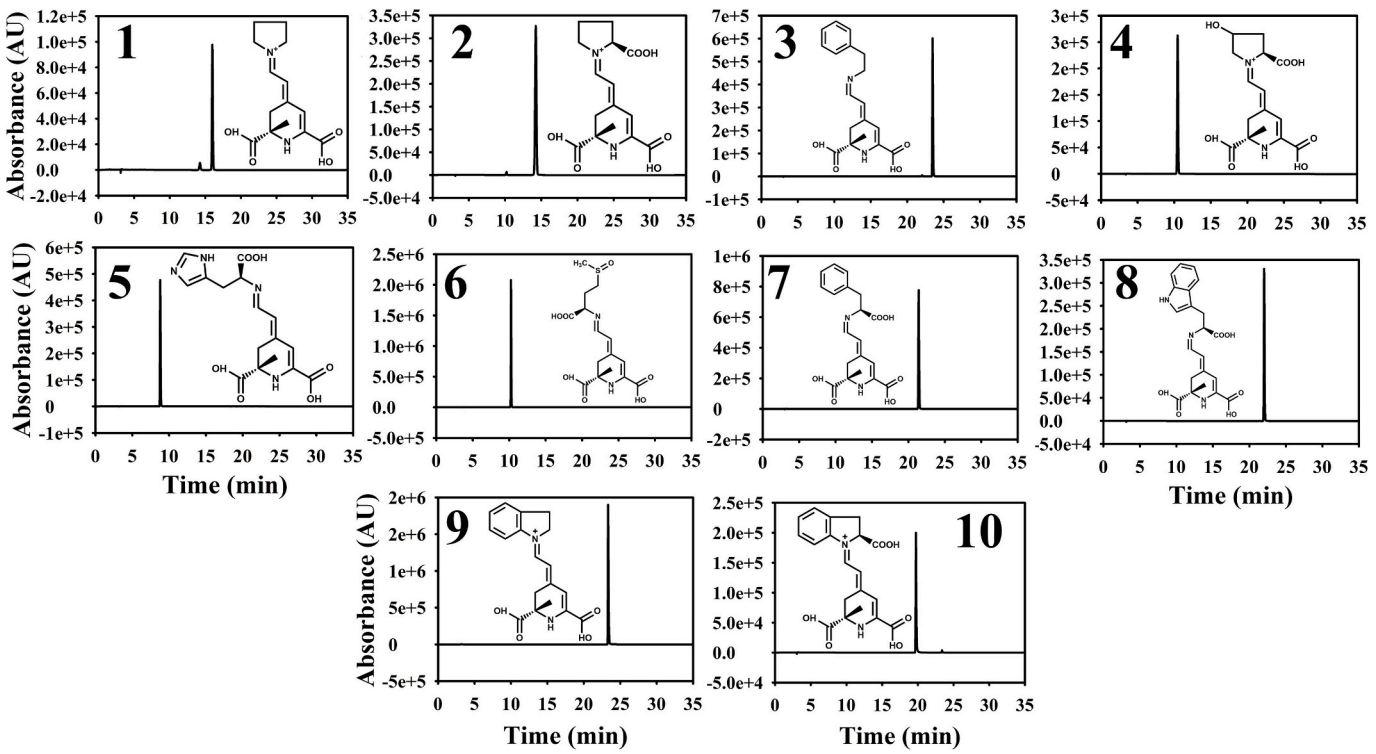

Fig. 6. Chemical structures and HPLC chromatograms of the methylated betalains synthesized in this work. The chromatograms at 480 nm for the methylated betaxanthins and at 536 nm for the methylated betacyanins are shown. (1) pyrrolidine-6-methyl-betaxanthin; (2) proline-6-methyl-betaxanthin; (3) phenylethylamine-6-methyl-betaxanthin; (4) hydroxyproline-6-methyl-betaxanthin; (5) histidine-6-methyl-betaxanthin; (6) methionine sulfoxide-6-methyl-betaxanthin; (7) phenylalanine-6-methyl-betaxanthin; (8) tryptophan-6-methyl-betaxanthin; (9) indoline-6-methyl-betacyanin; (10) indoline COOH-6-methyl-betacyanin.

Table 2
Comparison of retention times (RT) of traditional, decarboxylated and methylated betalains derived from the formation of imines between the same amines and different structural units.

Common name	Traditional betalain	RT (min)	Decarboxylated Betalain	RT (min)	Methylated betalain	RT (min)	References
–	Pyrrolidine-betaxanthin	15.20	Pyrrolidine-6-decarboxy-betaxanthin	16.00	Pyrrolidine-6-methyl-betaxanthin	15.95	[18,26]
Indicaxanthin	Proline-betaxanthin	12.65	Proline-6-decarboxy-betaxanthin	13.85	Proline-6-methyl-betaxanthin	14.23	
–	Phenylethylamine-betaxanthin	22.10	Phenylethylamine-6-decarboxy-betaxanthin	24.00	Phenylethylamine-6-methyl-betaxanthin	23.48	
Miraxanthin I	Methionine sulfoxide-betaxanthin	8.67	Methionine sulfoxide-6-decarboxy-betaxanthin	9.72	Methionine sulfoxide-6-methyl-betaxanthin	10.16	
–	Phenylalanine-betaxanthin	20.08	Phenylalanine-6-decarboxy-betaxanthin	21.20	Phenylalanine-6-methyl-betaxanthin	21.36	
–	Tryptophan-betaxanthin	20.28	Tryptophan-6-decarboxy-betaxanthin	21.97	Tryptophan-6-methyl-betaxanthin	22.03	
–	Indoline-betacyanin	22.10	Indoline-6-decarboxy-betacyanin	24.40	Indoline-6-methyl-betacyanin	23.32	
–	Indoline COOH-betacyanin	19.17	Indoline COOH-6-decarboxy-betacyanin	21.00	Indoline COOH-6-methyl-betacyanin	19.72	

betalains, demonstrating the potential of using alternative precursors as a strategy for the targeted synthesis of new betalains with structural modifications on the betalamic ring.

3.6. Spectroscopic characterization of methylated betalains: Color

Methylated betalains showed different coloration, from the yellow-orange of betaxanthins obtained from linear amines, to the red-violet of betacyanins obtained from indoline-derived amines. Analogously to the case with betalamic acid in traditional betalains [5] and with 6-decarboxy-betalamic acid in decarboxylated betalains [18], 6-methyl-betalamic acid acts as the chromophoric unit responsible for the color of these pigments. The nitrogen atoms in this substructure participate in the electron resonance system with the conjugated double bonds to yield its coloring properties to the pigment [5]. As part of the spectroscopic characterization of the new molecules, their absorption spectra were recorded (Supplementary Fig. S9) with wavelengths of maximum absorbance (λ_{max}) ranging from 474 to 530 nm (Table 4). Measurements were performed using water as solvent.

The molar extinction coefficient (ε) value obtained for 6-methyl-betalamic acid was 28,000 M^{-1} cm^{-1} at a wavelength of 432 nm. From this data, the ε values of the ten methylated betalains were calculated by end-point degradation reactions (Table 4). All compounds presented high values ranging from 45,000 to 68,000 M^{-1} cm^{-1}. The 6-methyl-betaxanthins, with λ_{max} ranging from 474 to 488 nm, presented ε values restricted to the range of 45,000 to 55,000 M^{-1} cm^{-1}. On the other hand, 6-methyl-betacyanins, whose λ_{max} were 524 and 530 nm, presented higher ε values with respect to 6-methyl-betaxanthins, being

Table 3
Experimental data obtained for the methylated betalains synthesized in this work. The retention time (RT) obtained by HPLC, the molecular formula, and the exact mass defined by HPLC-ESI-Q-TOF-MS for each molecule are shown.

Betalain	HPLC RT (min)	Chemical Formula	TOF exact mass		
			Theoretical (*m*/*z*)	Experimental (*m*/*z*)	Δppm
(1) Pyrrolidine-6-methyl-betaxanthin	15.95	$C_{14}H_{19}N_2O_4^+$	279.1339	279.1341	0.71650201
(2) Proline-6-methyl-betaxanthin	14.23	$C_{15}H_{19}N_2O_6^+$	323.1238	323.1236	−0.61895781
(3) Phenylethylamine-6-methyl-betaxanthin	23.48	$C_{18}H_{20}N_2O_4$	329.1496	329.1499	0.91143966
(4) Hydroxyproline-6-methyl-betaxanthin	10.46	$C_{15}H_{19}N_2O_7^+$	339.1187	339.1195	2.35905599
(5) Histidine-6-methyl-betaxanthin	8.79	$C_{16}H_{18}N_4O_6$	363.1299	363.1303	1.10153419
(6) Methionine sulfoxide-6-methyl-betaxanthin	10.16	$C_{15}H_{20}N_2O_7S$	373.1064	373.1065	0.26802006
(7) Phenylalanine-6-methyl-betaxanthin	21.36	$C_{19}H_{20}N_2O_6$	373.1394	373.1397	0.80398907
(8) Tryptophan-6-methyl-betaxanthin	22.03	$C_{21}H_{21}N_3O_6$	412.1503	412.1516	3.15418914
(9) Indoline-6-methyl-betacyanin	23.32	$C_{18}H_{19}N_2O_4^+$	327.1339	327.1340	0.30568523
(10) Indoline COOH-6-methyl-betacyanin	19.72	$C_{19}H_{19}N_2O_6^+$	371.1238	371.1242	1.07780746

Table 4
Absorbance and fluorescence spectroscopy data obtained for methylated betalains. Absorbance parameters given are: wavelength of maximum absorbance (λ_{max}), molar extinction coefficient (ε), and spectrum amplitude, measured at half of the maximum absorbance value. For fluorescence, parameters given are: wavelength of maximum excitation ($\lambda_{ex\ max}$) and maximum emission ($\lambda_{em\ max}$), Stokes shift, fluorescence intensity, and amplitude of the excitation and emission spectra, measured at half of the maximum fluorescence intensity values.

Betalain	Absorbance			Fluorescence					
	λ_{max} (nm)	ε (M^{-1} cm^{-1})	Width (nm)	$\lambda_{ex\ max}$ (nm)	$\lambda_{em\ max}$ (nm)	Stokes shift (nm)	Intensity (%) [a]	Ex width (nm)	Em width (nm)
(1) Pyrrolidine-6-methyl-betaxanthin	476	47,000	64	479	513	34	39.9	69	61
(2) Proline-6-methyl-betaxanthin	486	53,000	58	488	513	25	176.4	64	59
(3) Phenylethylamine-6-methyl-betaxanthin	476	53,000	54	477	505	28	141.1	62	62
(4) Hydroxyproline-6-methyl-betaxanthin	488	52,000	48	489	513	24	294.6	59	69
(5) Histidine-6-methyl-betaxanthin	478	55,000	50	478	507	29	417.5	58	60
(6) Methionine sulfoxide-6-methyl-betaxanthin	478	47,000	56	478	506	28	282.0	57	61
(7) Phenylalanine-6-methyl-betaxanthin	478	50,000	56	478	507	29	260.1	59	60
(8) Tryptophan-6-methyl-betaxanthin	474	45,000	60	477	509	32	12.02	68	65
(9) Indoline-6-methyl-betacyanin	524	67,000	72	524	574	50	33.8	77	90
(10) Indoline COOH-6-methyl-betacyanin	530	68,000	72	525	577	52	68.3	76	87

[a] Relative fluorescence intensity values are calculated in relation to dopaxanthin.

67,000 M^{-1} cm^{-1} for indoline-6-methyl-betacyanin and 68,000 M^{-1} cm^{-1} for indoline COOH-6-methyl-betacyanin.

The results obtained allow the distinction of two different groups of pigments according to their spectroscopic properties: the 6-methyl-betaxanthins, yellow in color, with lower λ_{max} and ε values; and the 6-methyl-betacyanins, violet in color, with higher values. 6-methyl-betaxanthins are formed by condensation of amines and amino acids without aromaticity in resonance with the nitrogen atoms, including cyclic compounds (pyrrolidine, proline and hydroxyproline), aromatic (phenylethylamine, phenylalanine, histidine and tryptophan) or with sulfur atoms (methionine sulfoxide), as it happens with traditional [5,27,28] and decarboxylated [18] betalains. On the other hand, 6-methyl-betacyanins contain aromatic resonance systems and cyclic structures fused to 6-methyl-betalamic acid, causing a distortion in the planarity of the resonance system of these pigments, similar to what happens in traditional [5,27,28] and decarboxylated [18] betalains. Therefore, the spectral properties of the new family of methylated betalains are analogous to the natural pigments and depend on the nature of the molecule that condenses with 6-methyl-betalamic acid.

3.7. Spectroscopic characterization of methylated betalains: Fluorescence

The novel 6-methyl-betalains are fluorescent, and their properties have been studied individually. First, the emission spectrum of each compound was analyzed by excitation at the maximum absorbance wavelength and then the excitation spectrum was recorded with the emission at the maximum wavelength determined (Fig. 7). The fluorescent properties of the ten methylated betalains are shown in Table 4. Considering the data obtained, it is observed that the 6-methyl-betaxanthins presented a range of values similar to those of traditional betaxanthins for maximum excitation (463–475 nm) and emission (506–515 nm) wavelengths [15,27]; and also to those of the decarboxylated pigments (457–487 nm and 502–516 nm for excitation and emission, respectively) [22]. In the same manner, 6-methyl-betacyanins presented a wavelength range similar to their natural analogs (521 to 529 nm, for excitation; 570 to 575 nm, for emission) [5,18]. It is observed how the presence of certain structural groups influences fluorescence intensity. Histidine-6-methyl-betaxanthin stands out as the pigment with the highest intensity value (417.5 % with respect to the reference compound, dopaxanthin) compared to the rest of the molecules analyzed. The pigment with the lowest fluorescence intensity was tryptophan-6-methyl-betaxanthin, with a value of 12.02 % in comparison to dopaxanthin. Regarding the 6-methyl-betaxanthins, it is observed that histidine-6-methyl-betaxanthin presents an intensity nearly four times higher than its traditional analog [5]. Proline-6-methyl-betaxanthin and pyrrolidine-6-methyl-betaxanthin, both structurally related, show that the extra carboxyl group (-COOH) causes an increase in fluorescence intensity. Similarly, phenylalanine-6-methyl-betaxanthin, an aromatic betalain, presents an additional carboxylic acid which increases its fluorescence intensity compared to phenylethylamine-6-methyl-

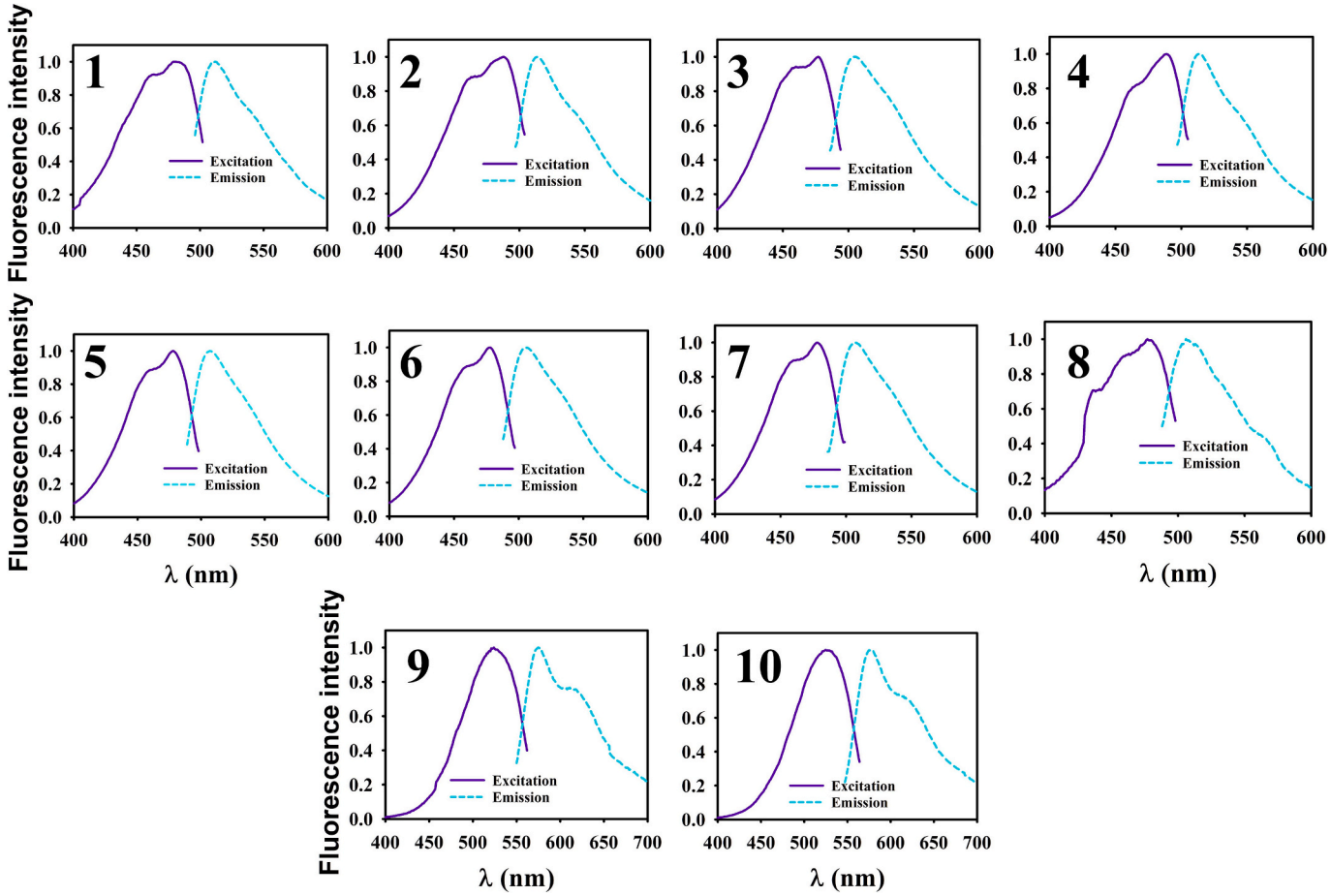

Fig. 7. Fluorescence spectra of the ten methylated betalains synthesized in this work. Excitation (solid lines) and emission (dashed lines) fluorescence spectra are shown for (1) pyrrolidine-6-methyl-betaxanthin; (2) proline-6-methyl-betaxanthin; (3) phenylethylamine-6-methyl-betaxanthin; (4) hydroxyproline-6-methyl-betaxanthin; (5) histidine-6-methyl-betaxanthin; (6) methionine sulfoxide-6-methyl-betaxanthin; (7) phenylalanine-6-methyl-betaxanthin; (8) tryptophan-6-methyl-betaxanthin; (9) indoline-6-methyl-betacyanin; (10) indoline COOH-6-methyl-betacyanin. The pigments were dissolved in water to a final concentration of 3 μM.

betaxanthin, as also occurs among its decarboxylated analogs [18]. Another prominent molecule is tryptophan-6-methyl-betaxanthin, with its fluorescence intensity being reduced due to the presence of an indole group, as it is also the case of the decarboxylated analog [18]. Regarding the 6-methyl-betacyanins, both indoline-6-methyl-betacyanin and indoline COOH-6-methyl-betacyanin presented reduced but close intensity values, the latter standing out with a value of 68.3 %, and both being more fluorescent than their traditional [5] and decarboxylated [18] analogs. Thus, from a structural standpoint, methylation at position 6 of betalamic acid may introduce electron-donating character, influencing the resonance system and altering its fluorescent properties [6] as observed for histidine-6-methyl-betaxanthin compared to its traditional analog. On the other hand, focusing on the side chain substituents, previous studies have shown that carboxyl groups can exert an electron-withdrawing effect on the resonance system, leading to an increase in fluorescence intensity [6] as observed here in the betalains derived from proline and phenylalanine compared to those derived from pyrrolidine and phenylethylamine, respectively.

3.8. Antioxidant activity of methylated betalains

The bioactive potential of the new molecules obtained was measured by evaluating their antioxidant activity by ORAC assays. The ten methylated pigments diluted in 75 mM sodium phosphate buffer, pH 7.4, protected the probe against peroxyl radicals (Fig. 8), and the results were obtained as Trolox equivalent antioxidant capacity (TEAC). The molecules with the highest TEAC values were indoline-6-methyl-betacyanin (4.2), tryptophan-6-methyl-betaxanthin (3.7), methionine sulfoxide-6-methyl-betaxanthin (3.5) and proline-6-methyl-betaxanthin (3.2). Although, in general, the values obtained were lower than those exhibited by other betalain families, the results showed that indoline-6-methyl-betacyanin presented higher antioxidant activity than its traditional analog [26]. Thus, the present work, added to previous studies on traditional [26] and decarboxylated [18] betalains, shows how structural modifications affect the antioxidant activity of these pigments and yields additional information to be taken into account for the design and production of new betalains with improved properties.

4. Conclusions

A novel chromophore and structural unit for betalain pigments has been obtained from the oxidative cleavage of α-methyl-DOPA: 6-methyl-betalamic acid. This is the first report on the incorporation of a functional group into the core structure of these pigments. Methylation of the betalamic ring alters the behavior of the synthetic pathway and can hinder the formation of the betaxanthin derived from the condensation between α-methyl-DOPA and 6-methyl-betalamic acid. This is used to favor the directed synthesis of other methylated betalains of interest. This approach enabled the synthesis of new betalamic pigments from α-methyl-DOPA, designed, produced, purified and characterized for the first time. The methylated betalains presented yellow (6-methyl-betaxanthins) or violet (6-methyl-betacyanins) coloration, with λ_{max} values

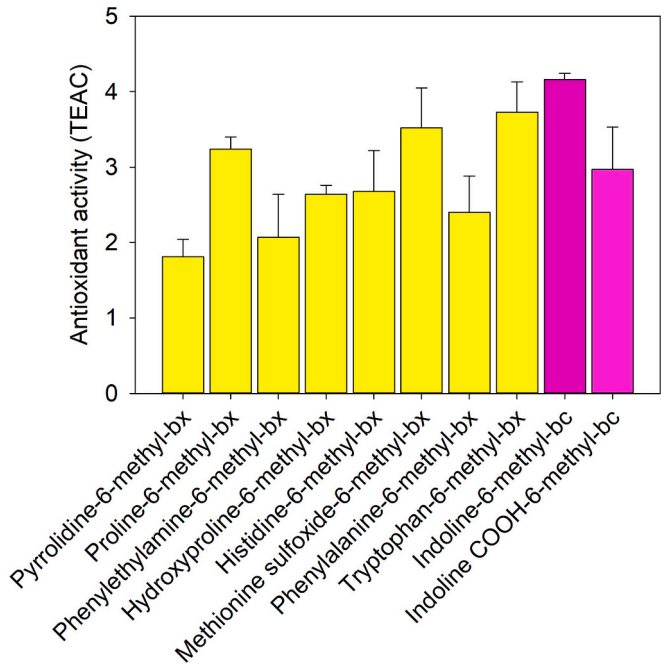

Fig. 8. In vitro antioxidant activity of methylated betalains measured by ORAC assay. Three independent assays were performed in each case, and the mean value is represented. Error bars represent the standard deviation. Bx, betaxanthin; Bc, betacyanin.

slightly higher than those defined for the two families described in nature. Likewise, the fluorescent intensity of the new methylated betalains has been analyzed, highlighting histidine-6-methyl-betaxanthin as the one showing the highest fluorescence. Methylated betalains show antioxidant activity, with indoline-6-methyl-betacyanin standing out as the most active in the ORAC assay. The results obtained contribute to the knowledge of the structure-activity relationship in the betalain family of compounds, by showing how the methylation of the betalamic ring can affect the spectroscopic properties or the antioxidant activity with respect to their traditional analogs. Although betalains are known for their low toxicity and good biocompatibility, the influence of structural modifications could drive the development of future studies exploring these aspects to assess their industrial feasibility. The development and characterization of methylated betalains, as well as other heterocycle-modified betalain pigments, expands the number of molecules potentially usable as fluorescent probes in bioimaging or as natural colorants with applications in the food and pharmaceutical industries. This study represents the beginning of the era of betalain core chemistry by modifying the structural unit of the pigments.

CRediT authorship contribution statement

Pedro Martínez-Rodríguez: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Luis Eduardo Contreras-Llano:** Investigation, Formal analysis, Conceptualization. **Diego José Pagán-López:** Investigation, Formal analysis. **María Alejandra Guerrero-Rubio:** Validation, Methodology, Investigation. **José Daniel Lozada-Ramírez:** Visualization, Validation, Supervision. **Samanta Hernández-García:** Writing – review & editing, Methodology, Investigation. **Fernando Gandía-Herrero:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Author contributions

F. G.-H., S. H.-G and J. D. L.-R. supervised the experiments and supervised and completed the writing. P. M.-R., L. E. C.-L., D. J. P.-L., S. H.-G., and M. A. G.-R. performed the experiments. F.G.-H. and P. M.-R. conceived the project, designed the experiments, analyzed the data, and wrote the manuscript with the contributions of all the authors. S. H.-G. agrees to serve as the author responsible for contact and ensures communication.

Funding sources

This work was supported by the Spanish Ministry of Science and Innovation, project PID2021-122896NB-I00 (MCIN/AEI/10.13039/501100011033/FEDER, UE). P. M.-R. holds a contract financed by Fundación Séneca (Spain) (21587/FPI/21).

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: F. G-H reports financial support was provided by Spain Ministry of Science and Innovation. P. M-R reports financial support was provided by Fundación Séneca. S. H-G reports a relationship with Betaelegans Biotech that includes: board membership. M.A. G-R reports a relationship with Betaelegans Biotech that includes: board membership. F. G-H reports a relationship with Betaelegans Biotech that includes: board membership. Conflict of Interest: Some of the authors launched in 2022 the company Betaelegans Biotech, a spin-off from the public academic institution Universidad de Murcia (Spain), primarily devoted to the synthesis of betalain standards. This has not biased or influenced the conception, analysis, or writing of the present research. The authors declare no other competing financial interest. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors are grateful to Dr. Alejandro Torrecillas (SAI, University of Murcia, Spain) for skillful technical assistance in mass spectrometry experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2025.108623.

Data availability

The 10 molecules characterized in this work have been deposited in the Pubchem database under the external ID “methylbetalainX”, making X reference to the numbering assigned throughout the present text. The studied reaction intermediates were also deposited under the external ID “secomethylDOPA1”, “secomethylDOPA2”, “methylbetalamic” and “methylmuscaflavin” for the molecules 2,3-seco-methyl-DOPA, 4,5-seco-methyl-DOPA, 6-methyl-betalamic acid and 6-methyl-muscaflavin, respectively.

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