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Synthesis of optically active tetrameric melanin intermediates by oxidation of the melanogenic precursor 5,6-dihydroxyindole-2-carboxylic acid under biomimetic conditions

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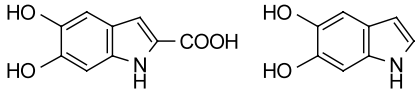
Abstract—In a recent work addressing the structural characterization of melanin pigments, we reported the isolation and characterization of trimeric oligomers of 5,6-dihydroxyindole-2-carboxylic acid (DHICA), a key intermediate in the biosynthesis of the dark brown eumelanins, by tyrosine catalyzed oxidation of the indole under biomimetic conditions. These oligomers feature atropisomerism and consequently, we wished to investigate chirality in such systems. Herein, we report two significant steps forward in this study: The isolation of a regiosymmetric DHICA tetramer by means of a model approach involving oxidation of the main DHICA dimer, namely 4,4′-biindolyl and the first resolution of eumelanin intermediates. This also allowed the absolute stereochemistry of the newly isolated tetramer to be defined by applying the exiton chirality method.

1. Introduction

Among natural pigments, the melanins[1a,b] occupy a unique position not only as a result of their involvement in human pigmentation,[1a,b] but also because of their extreme structural complexity[1c,d] which, combined with their unfavorable chemical-physical properties, prevents the definition of a structural model.[1b]

To date, the isolation and characterization of oxidative polymerization products arising from in vitro biomimetic oxidation of the pigment precursors, namely 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), proved to be the most promising approach in melanin structural investigation.[2]



By means of such a biomimetic approach, a number of melanin intermediate[2] were completely characterized, allowing us to outline structural hypotheses for melanins and particularly for DHICA-derived eumelanins, the dark brown pigments responsible for the diversity of skin, hair and eye color in man and other mammals,[1] which are likely to be made up of a mixture of linear polyindolyl oligomers[3–5] (Fig. 1). Among these dimers 4,4′-, 4,7′- and 7,7′-biindolyl **1**–**3** were identified

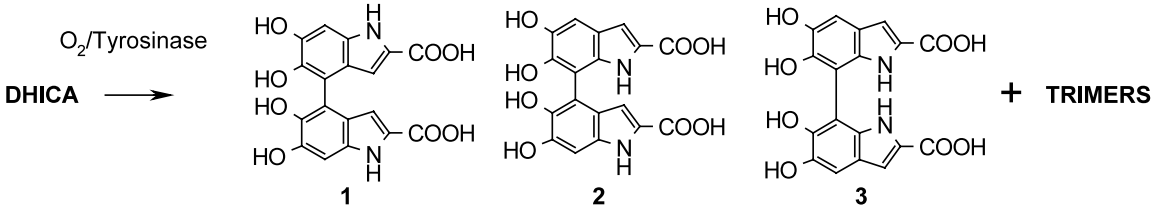


Figure 1. Oligomeric products arising from biomimetic DHICA oxidation: dimers 4,4′-biindolyl,[2d] 4,7′-biindolyl,[2c] 7,7′-biindolyl[2b] and trimers.[2a]

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In a recent work addressing eumelanin structural investigation, we reported the isolation of all possible regioisomeric linear trimers of DHICA.[2a] These were obtained by biomimetic tyrosinase/O_2 oxidation of DHICA leading to a complex reaction mixture which comprises other minor components. Indeed, MALDI MS analysis of the crude mixture revealed, beyond the presence of the peak at 576 m/z relative to trimeric constituents, a significant peak at 767 m/z which has to be ascribed to a tetrameric component.[3c,d]

Structural characterization of the regioisomeric trimers proved the existence of a significant rotational barrier, along DHICA–DHICA, linkages responsible for the dissymmetry of the molecule.[2a] Thus, from a stereochemical point of view, DHICA oligomers are analogous to *para*-polyaryls, which exhibit attractive structural features, such as helicity, and other connected unusual chemical–physical properties. Among these, optical activity is expected as consequence of the chiral nature of the oligomers.

Because of melanin involvement in skin photoprotection,[6] interest in such dissymmetric DHICA oligomers found further motivations, beyond the structural investigation, which derives from the non linear optical properties of melanin intermediates and their potential connection with the pigment functional role.

In light of this we furthered the melanin structural investigation with the double aim of isolating higher oligomers after trimers (i.e. MALDI MS-detected tetrameric products) and to determine optical activity, by means of CD spectroscopy, of the main melanin intermediates.

To surmount the difficulties connected with the low yields[2a] and the high number[7] of intermediates present in the DHICA tyrosinase/O_2 oxidation mixture, we assumed that tetrameric species mainly originated from 4,4′-biindolyl **1**, and thus focused on products arising from oxidative polymerization of this dimer. This allowed the complexity of the mixture to be reduced drastically without significant loss in the validity of the model, 4,4′-biindolyl being the main species in the reaction mixture for a significant time before trimer formation.[2,8]

2. Results and discussion

In order to obtain **1** in adequate amounts for investigative purposes, oxidative dimerization of DHICA proved to be the most fruitful approach. A number of different chemical and enzymatic oxidizing systems were tested. Among these, aerobic DHICA oxidation in aqueous buffer at pH 8.0 in the presence of cupric ions allowed **1** to be obtained in near pure form in 44% yield after acidic precipitation. Both pH and DHICA/Cu^{2+} molar ratio appear to be critical for driving the coupling at position 4 (Table 1) which probably involves the formation of a complex between the metal ion and DHICA.[2b]

Preliminary explorations of the oxidative polymerization of **1**, were thus performed by means of reverse phase HPLC analysis of the crude reaction mixtures. The reactions were carried out under conditions mimicking biological environments, i.e. aqueous buffers at pH 7.4, enzymatic catalyzed oxidation, thermostat bath at 37°C. The enzymatic oxidant systems chosen were tyrosine/O_2 and peroxiadase/H_2O_2, whose involvement in melanogenesis is so widely documented in literature,[1,9] to sustain their common use for in vitro biomimetic oxidation of melanogenic precursors.[1–4]

Chromatographic analysis showed that both tyrosinase and peroxidase were able to promote the oxidation of **1**. Dimer consumption in the tyrosinase-catalyzed oxidation was very slow, but the product pattern was more defined than in the case of peroxidase. It has been possible to obtain optimal conditions for the tyrosinase-catalyzed oxidation of **1** both in terms of product yields and reaction rate via the addition of DOPA (0.5% with respect to the dimer). Indeed, nearly complete consumption of the dimer was observed in the first 2 h of the reaction with concomitant formation of three distinct products[10] having a retention time higher than that of **1**. The products are indicated with labels **I–III** in the chromatogram (Fig. 2) relative to a reaction time of 45 min when the product yield reached the maximum.

Chemical oxidations of **1** were also performed using potassium ferricynide as oxidant and the cupric ions/O_2 system. These attempts were unfruitful, leading only to rapid consumption of the substrate with no concomitant formation of any defined product pattern.

Table 1. Yields of dimer **1** obtained by oxidation of DHICA in the presence of cupric ions

Oxidant	O_2/Cu^{2+} (pH 6.5) DHICA/Cu^{2+} molar ratio			O_2/Cu^{2+} (pH 8.0) DHICA/Cu^{2+} molar ratio			K_3FeCN_6 (pH 6.5)	$NaIO_4$ (pH 6.5)
	1/0.5	1/1	1/2	1/0.5	1/1	1/2	–	–
1% yield[a]	50	60	55	70	60	50	20	18

[a] Determined by HPLC at: 15 minutes (pH 6.5) and 10 (pH 8.0) reaction time. Other reaction condition as in Section 4.

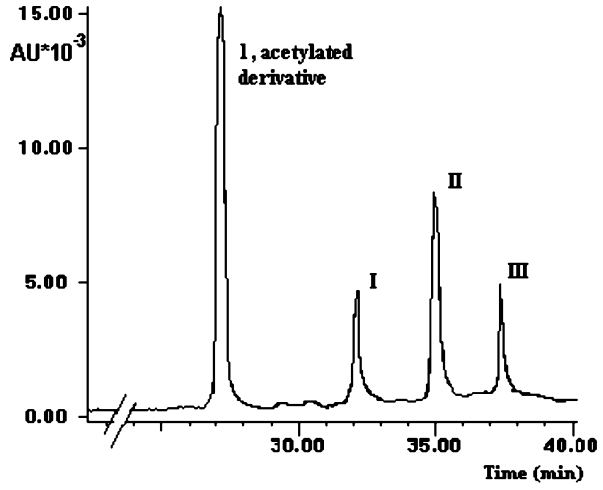


Figure 2. HPLC elution profile of the products arising by tyrosinase-catalyzed oxidation of dimer **1**, at 45 min of reaction, the acetylated derivative of **1** was identified by coinjection with an authentic sample. UV detection at 280 nm, see Section 4 for details.

Cooxidation of DHICA–**1** at 1:1 molar ratio presented a similar dependence on the oxidizing system shown by oxidation of **1**, giving, in the case of tyrosinase-catalyzed oxidation, four main products, which proved to be the trimers presenting the highest yields among products isolated from DHICA oxidation.[2a] This evidence also contributes to sustain the working hypothesis of a primary role of **1** in DHICA oxidative polymerization.

Attempts to isolate the products **I**–**III** by preparative HPLC presented some difficulties connected with their high tendency to oxidize and also to interconvert at temperatures as low as 70°C. Ethyl acetate extraction of the crude oxidation mixture after mild acidification to pH 4.0, and acetylation of the organic soluble fraction gave a brownish oil, whose purification by preparative reverse phase HPLC allowed each compound to be obtained as the pure acetylated derivative. Chromatographic analysis of the free and acetylated products established the retention of a relative elution order after acetylation, thus the same notation (i.e. **I**, **II**, **III**) will be adopted for both.

The mass spectra of all of the three acetylated derivatives presented the same value for the molecular ion at m/z 1103 ($M+H^+$), consistent with a structure consistent with a DHICA tetramer. The ^1H NMR spectra of the products **I** and **III** presented the common feature of having only three signals in the aromatic range while in the same region of the spectrum of **II**, six signals were distinguishable (Fig. 3). These data coupled with the observed temperature-dependent inter-conversion of the non-acetylated products suggested that compounds **I**–**III** were different atropisomers of the linear tetramer 4-4′:7′-7″:4″-4‴-quaterindole of DHICA.[11] Thus, in consideration of the chemical neighbors of each proton, the spectral data of both **I** and **III** are consistent with the structures **4** and **6**, while structure **5** may be assigned to product **II**. In particular, in structures **4** and **6** the protons H3 and H3‴ (like H3′ and H3″) are chemically equivalent and therefore exhibit identical chemical shifts, while **5** does not possess chemically equivalent protons and thus in the aromatic region of the spectrum, beyond the two signals for H7 and H7‴, four distinct signals are present (Fig. 3B).

Total signal assignment was made possible by analysis of the ^{13}C spectra and carbon–proton HMQC and HMBC spectra. Contacts between $H7^i$ and $C6^i$, $C8^i$ and $C9^i$ allowed the identification of signals for carbons belonging to the external units, while contacts between $H3^i$ and $C2^i$, $C5^i$ and $C9^i$ allowed nearly all carbon signals to be assigned. A small coupling between protons H3 and H7 led to the total assignment of proton signals for **4** and **6**, and the NOE contact between H3″ and H3‴ also allowed the proximity of the hydrogen at δ 6.72 with the one at δ 6.94 in **5** to be ascertained.

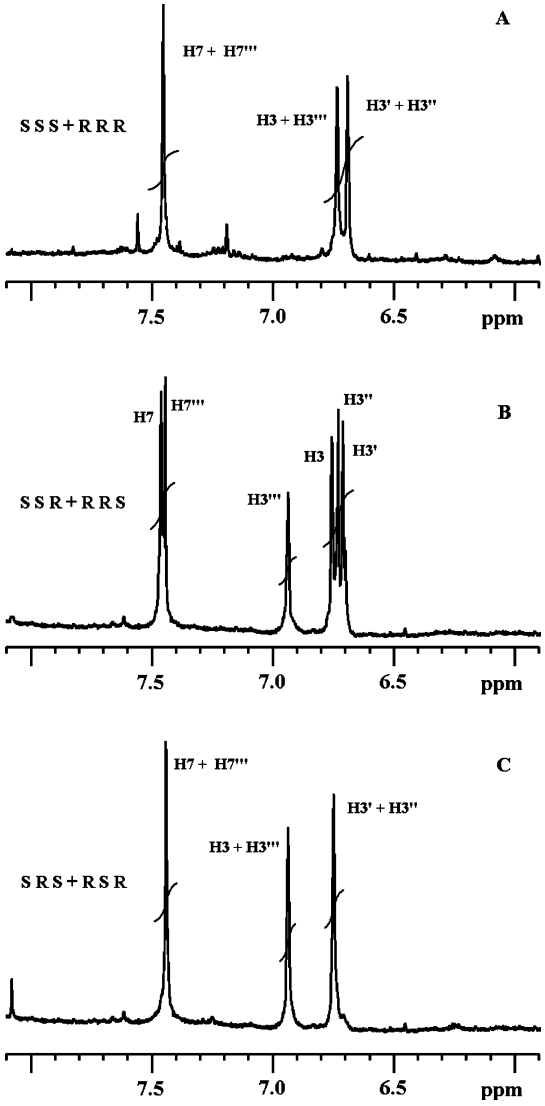
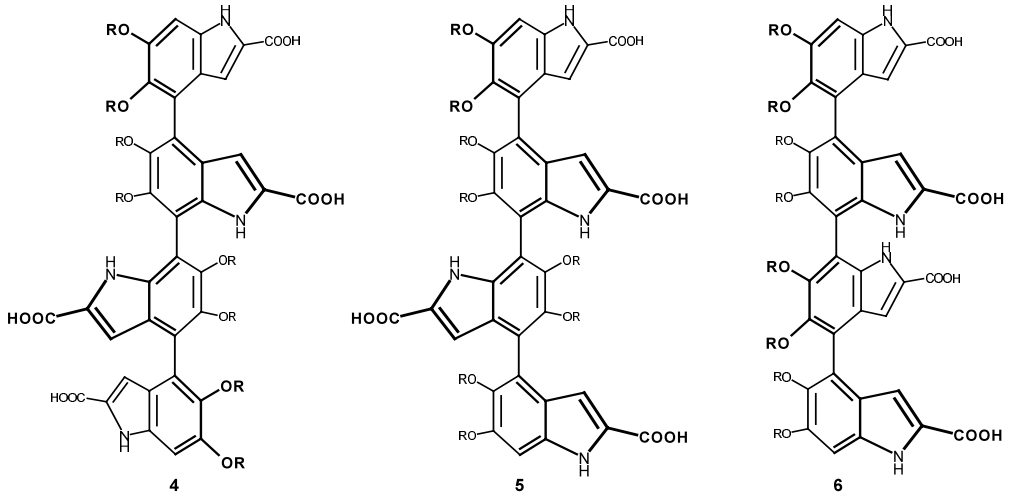


Figure 3. Aromatic region of the ^1H NMR spectra of **4**–**6**A–C respectively. Signals are labeled and configurations are indicated to underline the relation between chemical shifts and relative configurations of biindolyl sub-units.



Finally the attribution of structures **4** and **6** respectively to **I** and **III** was realized, as previously reported,[2a] by evaluation of NOE contact patterns between protons on positions 3 of external DHICA sub-units and protons bond to nitrogen atoms of internal DHICA subunits.

Like trimers,[2a] atropisomers **4**–**6** present discrete variations in the chemical shifts of the hydrogens H3 on the external indole units. This must be ascribed to a summation of shielding and deshielding effects arising from the internal indole units. In detail, starting from an external DHICA unit, because of the intramolecular distances, the indole linked at position 4 always exerts a shielding effect upon proton H3, the third indole in the chain may exert a moderate deshielding influence if the configurations of the two consecutive biindolyl subunits are opposite, while the effect of the fourth indole unit is negligible. This phenomenon, also observable in analogous polynaphthyl linear chains,[12] further contributes to sustain the relative configurations of tetramers derived on the basis of NOE effects.

Geometry optimization, by the use of molecular modeling software (hyperchem), showed that the dihedral angle between contiguous indole units has an absolute mean value of about 47°22′, which is very similar to that previously observed for DHICA trimers.[2a] This value is significantly smaller than the value of 110°, which is the theoretical zero point at which the sign of the exciton split of the CD Cotton effect changes from positive to negative for polyaryls featuring right-handed screwness.[13]

The absolute stereochemistry of compounds **1**–**6** was thus established by applying the exiton chirality method.

In order to obtain the pure enantiomers of **1**–**6**, racemate separation was performed by means of chiral HPLC. Products **4** and **6** were resolved using aqueous formic acid-isopropanol mixture as the mobile phase, while separation of enantiomers of **5** required a more complex eluent made up of a water-saturated 1-*n*-octanol: methanol:formic acid ternary mixture. Indeed, the octanol-based eluent also allowed the separation of **4** and **6**, but retention times were respectively 150 and 260 min at a flow rate of 0.5 mL/min, this led us to perform the enantiomer separation of **4** and **6** under different elution conditions. Enantioseparation was also performed for the acetylated derivatives of dimers **1**–**3** (also numbered **1**–**3** in the following) to obtain data about the chiral units of higher oligomers and to start up a CD spectra library for future investigations on natural[3b] and synthetic[3c] melanin pigments. After separation, the enantiomeric purity was determined by HPLC and proved to be at least 98% for all enantiomers obtained.

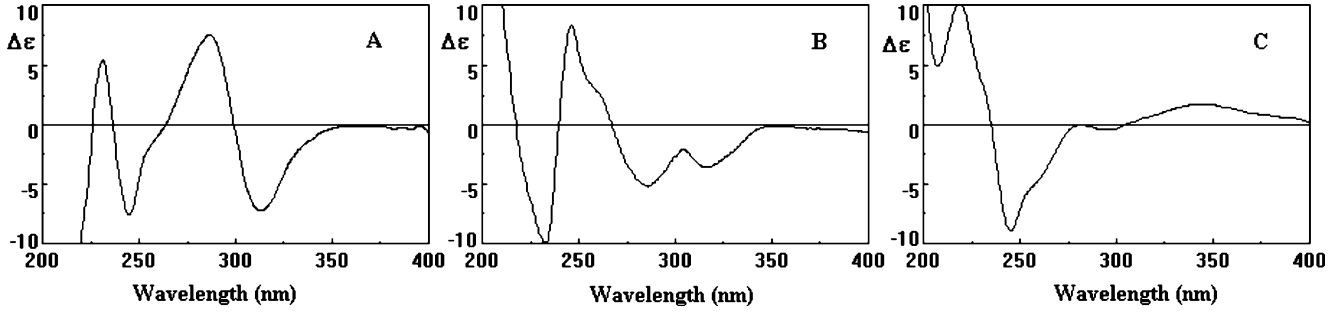


Figure 4. CD spectra of the first eluted enantiomer of dimers **1** (A), **2** (B) and **3** (C). Spectra were obtained at 25°C, UV absorbance at 304 nm were 0.23, 0.24 and 0.24, respectively.

Application of the exiton chirality method[14] was carried out on the base of transition moment directions reported in literature.[15,16] The assignment of absolute configuration was thus realized on the base of Cotton effect at 230 nm relative to the $1B_b$ transitions featuring a polarization nearly overlapping with the pseudosymmetry long axis of indole.[16] This transition is expected at 220 for underivatized indole, but the presence of an oxygen atom linked at position 6 of the indole may significantly alter the absorption maximum.[16] The choice of using the Cotton effect at 230 nm for configuration assignment, follows also the observation that this region of CD spectra of products **1**–**6** presents, with some intensity differences, a clear bisignated effect (Figs. 4 and 5) free of superimposed monosignated effects as, for example, in Fig. 4B. CD spectra were taken also of not acetylated dimers, obtaining only irrelevant changes in the appearance of the spectrum.

The negative Cotton effect at 230 nm, featured in the spectra in Fig. 4, indicates a left-handed screwness between the two indole pseudosymmetric long axes of the first eluted enantiomers of **1** and **3**, which are therefore respectively the (4-4′*R*)-2,2′-dicarboxy-5,5′,6,6′-tetraacetoxy-4,4′-biindole and (7-7′*R*)-2,2′-dicarboxy-5,5′,6,6′-tetraacetoxy-7,7′-biindole. In contrast, the first eluted enantiomer of dimer **2** presents right-handed screwness and thus possesses *S* configuration.

Whilst the CD spectra of tetramers are somewhat more complex, features relative to the dimer building blocks are recognizable. In light of the relative configuration deduced from the NMR spectra it has been possible to assign the absolute configuration to the first eluted enantiomer of the three tetramer racemates as reported in Table 2. In particular, the spectrum of the first eluted enantiomer of **4** (Fig. 5A) presents a negative Cotton effect and, with the NMR data being consistent with the helical structure (Fig. 3A), indicates the enantiomer is the (4-4′*S*,7′-7″*S*,4″-4‴*S*)-5,5′,5″,5‴,6,6′,6″,6‴-octaacetoxy-2,2′,2″,2‴ - tetracarboxy - 4,4′:7′,7″:4″,4‴ - quaterindole. Evaluation of $\Delta\varepsilon$ strengths at 248, 280 and 312 in the spectra of dimers **1** and **3** and of tetramer **4** allowed us to confirm that, as expected from the theory,[17] spectrum 5A reflects the summation of pairwise contribution of every possible bischromophoric interaction in the tetramer, that are, the internal 7-7′-biindolyl subunit and the external ones 4-4′-biindolyl. The same additivity principle also holds for the enantiomers of **5** and **6** (Fig. 5B–C).

Finally, it is interesting to note how, because of the additivity principle[17] and relative configuration of biindolyl sub-units known from NMR data, tetramer stereostructure assignments provided indirect confirmation of the matching between the relative elution order of the enantiomers of **1** and **3**.

3. Conclusions

Isolation of tetramers **4**–**6** and their stereochemical structure definition represents a significant step forward in the field of melanin structure investigation for two main reasons: Firstly, products **4**–**6**, together with previously isolated trimers conclusively validate the structural model of eumelanin as consisting of a mixture of linear DHICA oligomers and secondly direct evidence has been produced for the non linear optical behavior of melanin intermediates, this is a key chemical-physical feature for a natural pigment present in light exposed tissues[1] and also involved in photoprotection.[6]

As for trimers,[2a] the λ_{max} of products **4**–**6** does not present significant differences from that of DHICA, confirming

Table 2. Symmetry group, absolute configuration and rotatory power of the first eluted enantiomer of **1**–**6**

	Dimer **1**	Dimer **2**	Dimer **3**	Tetramer **4**	Tetramer **5**	Tetramer **6**
Symmetry group	*C*2	*C*1	*C*2	*C*2	*C*1	*C*2
Absolute configuration	*R*	*S*	*R*	*R*,*R*,*R*	*S*,*R*,*R*	*R*,*S*,*R*
[a]$[\alpha]_D^{25}$	−35.3	+42.4	−38.8	−54.6	−20.8	−32.2

[a] (*c* 1.0, EtOH).

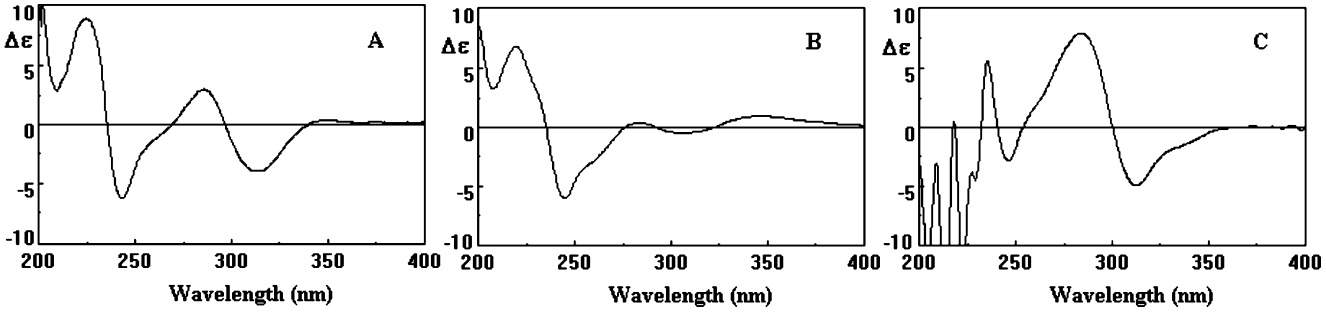


Figure 5. CD spectra of the first eluted enantiomer of tetramers **4** (A), **5** (B) and **6** (C). Spectra were taken at 25°C, UV absorbance at 304 nm were 0.20, 0.20 and 0.19, respectively.

the absence of extended conjugation of the indole units in such linear chains and sustaining the hypothesis[2a] that the black–brown color of eumelanin is a consequence of randomly disposed polarizing domains. Experiments are now in progress to investigate melanin interaction with linearly polarized light in order to validate this hypothesis.

Beyond for the advancement of melanin structural and functional investigation, DHICA oligomers also appear attractive as a result of their structural nature, closely connected with that of polyaryls linking at positions 1–4,[18] which suggests potential applications in material sciences.[19]

Finally, characterization of products **4–6**, providing ^{1}H and ^{13}C spectral data of the main tetrameric DHICA oligomers, completed the NMR library of the MALDI MS-detectable constituents of the eumelanin pigment.

4. Experimental

4.1. General

UV spectra were recorded on a Beeckman DU 640; CD spectra were obtained using a Jasco J-715/150S spectropolarimeter at 25°C. MALDI mass spectra were taken on a Reflex time-of-flight mass spectrometer, operating in positive linear mode; ions were formed by a pulsed UV laser beam (nitrogen laser, $\lambda = 337$ nm); 2,5-dihydroxybenzoic acid was used as matrix. For FAB MS measurements, glycerol was used as matrix. ^{1}H and (^{13}C) NMR spectra were recorded 400.1 (100.6) MHz or 200.2 (50.1) at an operating temperature of 298 K. COSY and ROESY experiments were run using a standard Bruker pulse program. 2D carbon-proton shift correlation experiments were carried out using a Bruker XHCORR microprogram with a D_3 delay corresponding to J values of 140 and 10 Hz. Tetramethylsilane was used as a reference standard. HPLC analyses were carried out on a Gilson apparatus equipped with a Gilson mod. 117 UV detector set at 280 nm. RP18 Spherisorb S50DS2 (4.0×250 mm, Phase Separation Ltd.) or Econosil C-18 10U (22×250 mm, Alltech) columns were used for analytical and preparative purposes, with flow rates of 1 mL/min and 15 mL/min, respectively. Mushroom tyrosinase (EC 1.14.18.1, *o*-diphenol:O_2 oxidoreductase, 2780 U/mg) and horseradish peroxidase (EC 1.11.1.7, donor:H_2O_2 oxidoreductase) type II (220 U/mg, RZ $E_{430}/E_{275} = 2.0$) were from Sigma. Hydrogen peroxide 30% (stabilized), potassium ferricyanide, cupric sulfate pentahydrate and all other chemicals were purchased from Aldrich. DHICA was synthesized by a literature procedure.[20] Glass distilled, deionized water was used for preparation of all solutions. Authentic samples of **1–3** were obtained as previously described.[2a,b] Molecular geometry optimizations were carried out with Hyperchem 5.0 Package produced by Hypercube Inc. (Waterloo, Ontario, Canada), 1997.

4.2. Oxidation of DHICA and dimer 1

A solution of the substrate (50 μmol) in phosphate buffer 0.1 M pH 7.4 (10 mL) was treated with (a) tyrosinase (400 units) under a stream of oxygen, (b) peroxidase (40 units)/0.3% hydrogen peroxide (220 μL), (c) potassium ferricyanide (26 μmol), (d) or (e) sodium periodate (13 μmol) varying amounts of cupric sulfate (13, 26 or 52 μmol and 26, 52 or 104 μmol for **1**). In the case (d) reactions were carried out in 0.5 M Tris buffer system, at pH 6.5 and 8.0. Enzymatic oxidations were performed in a thermostatic bath regulated at 25°C. Reactions were stopped for HPLC analysis, by addition of an excess of sodium borohydride. The mixture was then acidified with 2 M HCl to pH 3, filtered through a 0.45 μm Millipore filter and analyzed using as 0.05 M ammonium citrate–0.4 M ammonium formate, pH 2.5 containing 6% acetonitrile as the mobile phase. Dimers an trimers were identified by coinjection with authentic samples. Co-oxidation DHICA–**1** in 1:1 ratio was performed as above containing the starting solution 25 μmol of both DHICA and **1**; in the case (d) cupric sulfate amounts were 19, 39 or 78 μmol.

4.3. Synthesis of 1

To a solution of DHICA (500 mg) in 0.5 M TRIS buffer, pH 8.0 (300 mL), saturated with oxygen gas, was added a solution of $CuSO_4$ (3 mL, 345 mg) under vigorous stirring. After about 10 min under oxygen bubbling, the mixture was reduced with an excess of sodium borohydride, and filtered twice over paper, subsequent acidification of the liquors led to precipitation of **1** (220 mg, 95% NMR grade).

4.4. Isolation of tetramers 4–6

A solution of **1** (384 mg) in 0.1 M phosphate buffer, pH 7.0 (100 mL) and DOPA (~1 mg), was saturated with oxygen gas and then added with tyrosinase solution (4 mL, 400 units/mL) under vigorous stirring. The course of the reaction was followed by HPLC using 0.05 M ammonium citrate, pH 2.5 containing 10% acetonitrile as the mobile phase. After about 15 min, the mixture was reduced with an excess of sodium borohydride, acidified with HCl to ca. pH 2 and extracted five times with an equal volume of ethyl acetate. The organic layers were dried over sodium sulfate and evaporated to dryness. The residue (about 100 mg) was acetylated with acetic anhydride/pyridine 20/1 and fractionated by preparative HPLC, using 0.4 M formic acid/methanol 6/4 as the mobile phase. The peaks eluted at 32, 35, and 38 min were collected and carefully evaporated to dryness to give pure tetramers 4 (18 mg), 5 (26 mg), 6 (24 mg) in the order.

4.4.1. (4-4′*R*,7′-7″*R*,4″-4‴*R*)- and (4-4′*S*,7′-7″*S*,4″-4‴*S*)-5,5′,5″,5‴,6,6′,6″,6‴ - Octaacetoxy - 2,2′,2″,2‴ - tetracarboxy-4,4′:7′,7″:4″,4‴-quaterindole, 4. Pale gray to greenish vitreous solid. UV(MeOH): λ_{max} 304, log(ε) 3.60. IR: ν_{max} (KBr)/cm^{-1} 3405, 1726, 1580, 1466, 750. FAB(M+H)$^{+}$: m/z 1103. HRMS calcd for

$C_{52}H_{39}N_4O_{24}$ (M^++H) 1103.1954, found 1103.1929. ^{1}H NMR (MeOH-d_4), δ (ppm) 6.69 (2H, s, H3′ and H3″), 6.73 (2H, d, $J=0.8$ Hz, H3 and H3‴), 7.45 (2H, d, $J=0.8$ Hz, H7 and H7‴), 1.98 (6H, bs C5′- and C5″-$OCOCH_3$), 2.04 (6H, bs C5- and C5‴-$OCOCH_3$), 2.12 (6H, bs, C6′- and C6″-$COCH_3$). 2.31 (6H, bs, C6- and C6‴-$OCOCH_3$); ^{13}C NMR (MeOH-d_4), δ (ppm): 20.99, 21.58, 21.86 (6×$OCOCH_3$), 31.25 (2×$OCOCH_3$), 109.11 (C7, and C7‴), 110.69 (C3 and C3‴), 110.80 (C3′ and C3″), 114.22 (C7′ and C7″), 124.76, 125.09 (C4, C4‴, C4′ and C4″), 127.32, 127.48 (C9, C9′ C9″ and C9‴), 132.22, 132.43 (C2, C2′, C2″ and C2‴), 135.68 (C5 and C5‴), 135.84 (C5′ and C5″), 136.94 (C8′ and C8″), 137.54, 138.22 (C6, C6′, C6″ and C6‴), 143.05 (C8 and C8‴), 166.18, 166.23 (4× COOH), 171.14, 171.24, 171.38, 171.48 (8×$OCOCH_3$).

4.4.2. (4-4′*R*,7′-7″*R*,4″-4‴*S*)- and (4-4′*S*,7′-7″*S*,4″-4‴*R*)-5,5′,5″,5‴,6,6′,6″,6‴ - Octaacetoxy - 2,2′,2″,2‴ - tetracarboxy-4,4′:7′,7″:4″,4‴-quaterindole, 5. Pale gray to greenish vitreous solid. UV(MeOH): λ_{max} 306 nm, log(ε) 3.50. IR: ν_{max} (KBr)/cm^{-1} 3409, 1729, 1580, 1464, 750. FAB(M+H)$^+$: m/z 1103. HRMS calcd for $C_{52}H_{39}N_4O_{24}$ (M^++H) 1103.1954, found 1103.1932. ^{1}H NMR (MeOH-d_4), δ (ppm): 6.70 (H, s, H3′), 6.72 (H, s, H3″), 6.75 (1H, d, $J=0.8$ Hz, H3), 6.94 (1H, d, $J=0.8$ Hz, H3‴), 7.44 (1H, d, $J=0.8$ Hz, H7‴), 7.45 (1H, d, $J=0.8$ Hz, H7), 1.98 and 1.99 (6H, s, C5′- and C5″-$OCOCH_3$), 2.02, 2.07, 2.11 and 2.12 (12H, bs, C5-, C5‴, C6′- and C6″-$OCOCH_3$), 2.31 and 2.32 (6H, s, C6- and C6‴-$OCOCH_3$). ^{13}C NMR (MeOH-d_4), δ (ppm): 20.99, 21.24, 21.58, 21.86 (6×$OCOCH_3$), 31.25 (2×$OCOCH_3$), 109.14, 109.18 (C7 and C7‴), 110.39 (C3), 109.50 (C3‴), 109.99 (C3′), 111.08 (C3″), 114.20, 114.27 (C7′ and C7″), 114.98, 115.04 (C7 and C7‴), 124.76, 125.00, 125.19 (C4, C4‴, C4′ and C4″), 127.30, 127.38, 127. 42, 127.48 (C9, C9′ C9″ and C9‴), 132.22, 132.28, 132.40, 132.47 (C2, C2′, C2″ and C2‴), 135.70, 135.75 (C5 and C5‴), 135.84, 136.00 (C5′ and C5″), 136.95, 137.00 (C8′ and C8″), 137.54, 137.99, 138.20, 138.29 (C6, C6′, C6″ and C6‴), 143.05, 143.09 (C8 and C8‴), 167.00, 167.10, 167.19, 167.24 (4×COOH), 171.01, 171.20, 171.38, 171.46, 171.52 (8× $OCOCH_3$).

4.4.3. (4-4′*R*,7′-7″*S*,4″-4‴*R*)- and (4-4′*S*,7′-7″*R*,4″-4‴*S*)-5,5′,5″,5‴,6,6′,6″,6‴ - Octaacetoxy - 2,2′,2″,2‴ - tetracarboxy-4,4′:7′,7″:4″,4‴-quaterindole, 6. Pale gray to greenish vitreous solid. UV(MeOH): λ_{max} 304, log(ε) 3.67. IR: ν_{max} (KBr)/cm^{-1} 3410, 1720, 1583, 1466, 748. nm FAB(M+H)$^+$: m/z 1103. HRMS calcd for $C_{52}H_{39}N_4O_{24}$ (M^++H) 1103.1954, found 1103.1927. ^{1}H NMR (MeOH-d_4), δ (ppm): 6.75 (2H, s, H3′ and H3″), 6.94 (2H, s, $J=0.8$ Hz, H3 and H3‴), 7.44 (2H, s, $J=0.8$ Hz, H7 and H7‴), 1.99 (6H, bs, C5′- and C5″-$OCOCH_3$), 2.04 (6H, bs, C5- and C5‴-$OCOCH_3$), 2.11 (6H, bs C6′- and C6″-$OCOCH_3$), 2.32 (6H, bs C6- and C6‴–$OCOCH_3$). ^{13}C NMR (MeOH-d_4), δ (ppm): 20.99, 21.58 (4× $OCOCH_3$), 31.19 (2×$OCOCH_3$), 31.25 (2×$OCOCH_3$), 109.21 (C7 and C7‴), 110.50 (C3 and C3‴), 110.59 (C3′ and C3″), 115.01 (C7 and C7‴), 124.26, 124.59 (C4, C4‴, C4′ and C4″), 127.30, 127.42 (C9, C9′ C9″ and C9‴), 132.29, 132.34 (C2, C2′, C2″ and C2‴), 135.45 (C5 and C5‴), 135.78 (C5′ and C5″), 136.90 (C8′ C8″), 137.74, 138.20 (C6, C6′, C6″ and C6‴), 143.15 (C8 and C8‴), 166.68, 167.20 (4×COOH), 171.21, 171.28, 171.34, 171.38 (8×$OCOCH_3$).

4.5. Resolution of enantiomers

Enantiomer separation was carried out by means of chiral phase HPLC, using a Supelco chiral phase column ((*R*)-3,5-dinitrobenzoylphenylglycinepropylsilyl (5 μ), 4.6 mm×25.0 cm) with double UV–vis detection at 280 and 300 nm and different elution condition as follows: flow 1 mL/min, mobile phase: 0.5% aqueous formic acid/isopropanol in ratios 6/4, 5/5, 4/6, 3/7 for respectively **1**, **2**, **3**, **4** and **6**; flow 0.3 mL/min, mobile phase: water-saturated methanol/*n*-1-octanol/formic acid 60/20/1 for **5**. Retention times for the first eluted enantiomers were in the order: 16, 13, 18, 11, 250, 20 min.

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7. The number of possible distinct regioatropisomers for a DHICA linear oligomer has been obtained by means of a combinatory approach, it is 2^{4n} for an oligomer of $N=2n+1$ DHICA units and $2^{(2n-1)}(2^{(2n-1)}+1)$ for an oligomer of $N=2n$ DHICA units.
8. The relative dimer yields were evaluated under different reaction conditions involving peroxidase/H_2O oxidation and tyrosinase catalyzed aerobic oxidation. The collected data showed, apart from small variations in the yield of **2**–**3**, that **1** constitutes at least 60% of oligomeric detectable species, before trimers formation, in any tested conditions.
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10. After enantiomer separation, tyrosinase-catalyzed oxidation of pure (*S*)-4,4′-biindolyl was also carried out obtaining only one enantiomer of **4** and **6**, namely the (*S*,*S*,*S*)- and the (*S*,*R*,*S*)-quaterindole.
11. As with trimers, the atropisomeric nature of the three isomers has been confirmed by dynamic ^{1}H NMR experiments on the underivatized species.[2a]
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