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Atropoisomeric melanin intermediates by oxidation of the melanogenic precursor 5,6-dihydroxyindole-2-carboxylic acid under biomimetic conditions

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Abstract—A great part of the current knowledge about the chemistry and the structure of melanins arises from in vitro studies involving oxidative polymerization of 5,6-dihydroxyindoles under biomimetic conditions. Previous studies carried out on the 5,6-dihydroxyindole-2-carboxylic acid (DHICA), a key intermediate in the biosynthesis of the dark brown eumelanins, had delineated a reaction pathway involving mainly the formation of the symmetric dimer 4-4′-biindolyl together with a lower amount of 4-7′-, 7-7′-3-4′-, 3-7′-coupled dimers. To further understand the melanin structure, we investigated oxidative polymerization of DHICA under conditions of relevance to biomimetic processes. Four new linear trimers in eight atropoisomeric forms have been isolated and characterized giving the first evidence for the chiral nature of these early oligomeric species. © 2002 Elsevier Science Ltd. All rights reserved.

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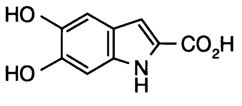
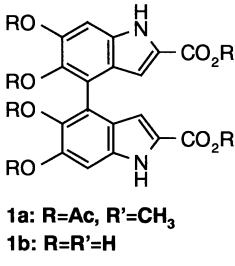
1. Introduction

Melanin research has traditionally stimulated academic and practical interest because of the key role of these pigments in skin pigmentation[1] and photoprotection[2] and their increasing exploitation in a variety of industrial application.[3] Extensive studies carried out over the past 60 years have produced considerable steps forward in all areas of melanin research, especially concerning the identification of their biogenetic precursor(s)[1] and elucidation of the early steps of melanogenesis.[1] At present, it seems likely to be established that eumelanin, the dark brown pigment responsible for the diversity of skin, hair and eye color in man and other mammals[4] and present in the ink of some marine invertebrates,[5] arises from oxidative polymerization of 5,6-dihydroxyindole-2-carboxylic acid (DHICA).[6–8] In spite of unabated interest and extensive efforts, a major gap still remains concerning the structure of this elusive and enigmatic pigment. The reason for this may be ascribed to the noteworthy difficulty connected with the isolation of pure pigment and to its high instability and insolubility in any solvent which prevent employment of the usual investigative tools, such as NMR spectroscopy and X-ray diffraction, for structural characterization.[1]

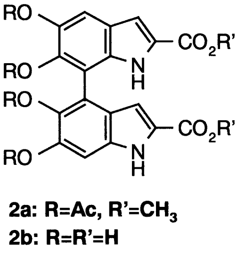
For these causes, the progress in unraveling the structure of melanin, and particularly eumelanin, stems, to a large extent, from the understanding of the mechanism of DHICA polymerization by way of structural characterization of the products formed by in vitro biomimetic oxidation of this melanogenic precursor.[1a,9] Some insights followed from the development of ad hoc procedures that lead to the isolation of DHICA dimers 4-4′-biindolyl (**1a**) and 4-7′-biindolyl (**2a**). This procedure involved the following steps: oxidation of DHICA by peroxidase/H_2O_2 system; reduction of the oxidation mixture with sodium dithionite; esterification of the ethyl acetate extractable fraction with anhydrous HCl saturated methanol; acetylation with acetic anhydride.[10] More recently, an improvement of this procedure allowed underivatized dimers 4-4′-biindolyl **1b**, **2b** and three new products, the 3-linked dimers **3**, **4** and the 7,7′-biindolyl **5**, by preparative HPLC isolation, to be obtained.[11] However, no significant information was obtained about the way of growth and the structure of higher



DHICA



1a: R=Ac, R'=CH$_3$
1b: R=R'=H



2a: R=Ac, R'=CH$_3$
2b: R=R'=H

Keywords: melanin; DHICA; atropoisomers; diastereoisomers.

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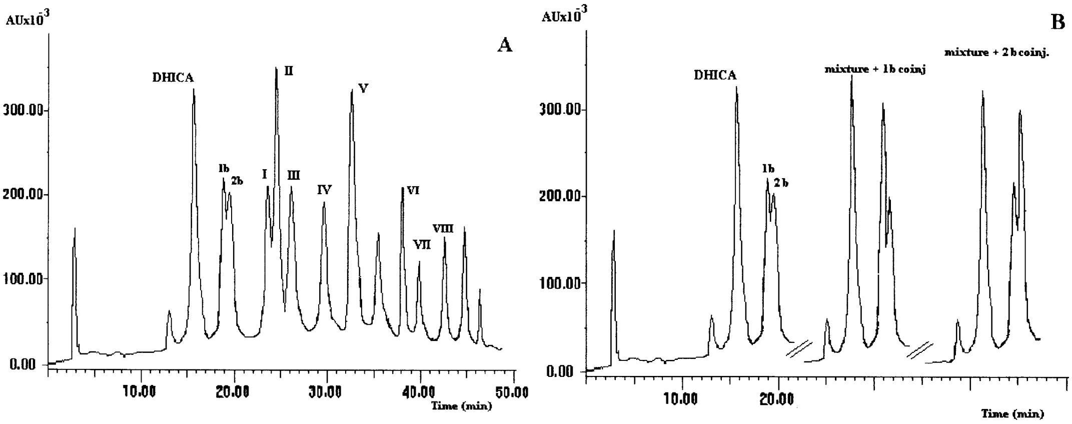
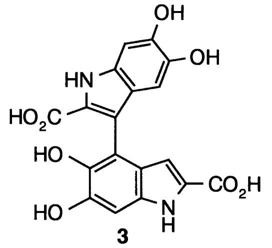


Figure 1. HPLC elution profile of the products arising by tyrosinase catalyzed DHICA oxidation at 10 min of reaction course (A); identification of products **1b** and **2b** by coinjection with authentic samples (B). UV detection at 280 nm, see Section 4 for details.



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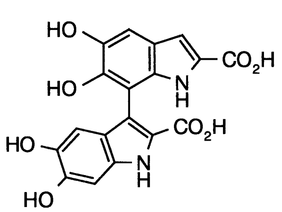

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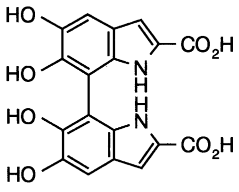

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oligomers and melanin intermediates. In this work, we report the isolation and characterization of the main products, after dimers, obtained by biomimetic in vitro DHICA oxidation, which revealed to be all the possible regioisomeric trimers.

2. Results and discussion

Enzyme-catalyzed as well as chemically promoted DHICA oxidations were first examined to achieve the double aim of furthering eumelanin structural investigation and finding an expedient synthesis for gram-scale amounts of dimers and higher oligomers production. In all the conditions examined, chemical oxidants produced a rapid DHICA consumption with concomitant formation of a dark brown precipitate, while enzymic catalyzed oxidations led to a well-defined product pattern as revealed by HPLC profiles. Careful analysis of the tyrosinase/O_2 oxidation mixture allowed identification of dimers **1b**, and **2b** by coinjection with authentic samples (Fig. 1B). The elution profile became very complex after the first 10 min of reaction time, presenting some 10 more distinguishable products among which some, indicated with labels I–VIII (Fig. 1A), appeared to prevail.

Because of the low yields and the high number of products, it has been difficult to isolate enough material to perform structural characterization of all the products. Nevertheless, compounds corresponding to peaks I–VIII have been isolated and characterized as acetylated derivatives. All the products isolated presented the same molecular weight of *m*/*z* 828 ($M+H^+$) and very similar ^1H and ^{13}C NMR spectra which allowed the attribution of a linear trimeric structure to all of them. On the basis of NOE and carbon proton correlation experiments, it has been possible to realize the complete assignment of the proton and carbon signals and to assume an atropoisomeric link between the constituents of the couples II–V, I–III, IV–VI, VII and VIII

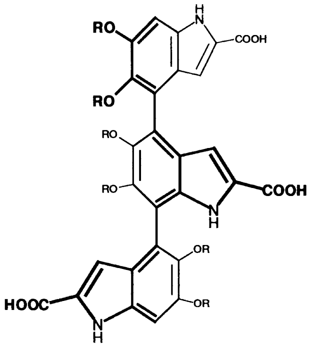
Table 1. Chemical shifts and NOE contacts of protons at positions three and on nitrogen of trimers **6**–**13**

Compound	δ (ppm) (protons exhibiting NOE contact)[a]					
	H3	H3′	H3″	NH	NH′	NH″
6	6.66 [**H3′**]	6.69 [**H3**]	6.62 [NH′]	12.09	11.05 [H3″]	11.90
7	6.80 [**H3′**, *H3″*]	6.70 [**H3**]	6.74 [*H3*, NH′]	12.00	11.10 [H3″]	11.85
8	6.69 [**H3′**]	6.75 [H3]	7.32	12.10	10.98 [NH″]	11.31 [NH′]
9	6.93 [**H3′**, *NH″*]	6.72 [**H3**]	7.32	12.05	11.03 [NH″]	11.45 [*H3*, NH′]
10	6.65 [NH′]	6.58 [NH″]	7.30	11.98	11.03 [H3]	11.12 [H3′]
11	6.90 [NH′, *NH″*]	6.61 [NH″]	7.30	12.00	11.05 [H3]	11.23 [*H3*, H3′]
12	–	6.62 [NH]	–	11.13 [H3′]	11.07	11.09
13	–	6.66 [NH]	–	11.20 [H3′, *NH″*]	11.10	11.19 [*NH*]

Chemical shift—for NH chemical shifts and NOE contacts determination experiments were run in DMSO-d_6, this allowed also the determination of small ^{H-H}J coupling among $H3^i$ and NH^i.

NOE contacts—ROESY experiments.

[a] Bold indicates strong contact, italic indicates very weak contact.



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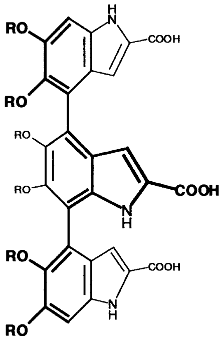

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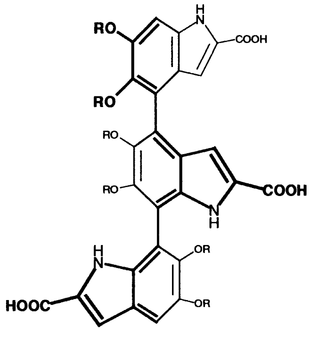

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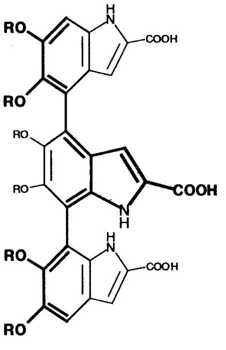

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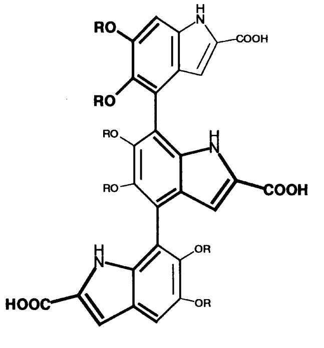

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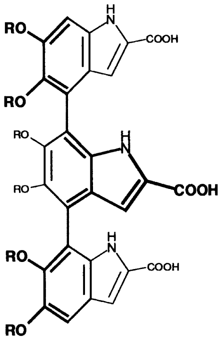

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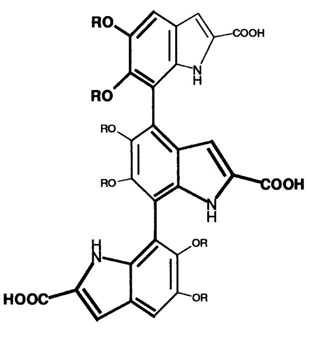

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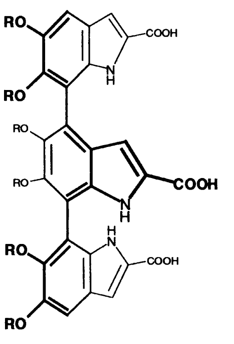

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R=-$COCH_3$ (Only one enantiomer for each atropoisomer is represented)

to which the structures **6–13** have been attributed respectively. The atropoisomerism has been confirmed by dynamic ^{1}H NMR experiments on the underivatized species. Line shape analysis at several temperatures, around coalescence (T_c=350 K), allowed calculation of the mean lifetime from which a free energy of activation of (21.48±−0.1 kcal/mol) was determined by straightforward application of the Eyring equation.[12]

The spatial arrangement of the indolic units for each constituent of the couple II–V has been derived from the NOE effect between H3 and H3″, which was observed for **7** and absent for **6**. In Table 1 are collected both the chemical shift and the NOE effect exhibited by H3^i and NH^i, of all the atropoisomeric trimers isolated. As expected, the contact intensity is minimal for protons (i.e. H3 and H3″ of **7**) whose distance is close to limit of observable NOE effect.[13] The chemical shifts of the H3^i range from 6.58 to 6.90 ppm depending on shielding effects of the indole ring systems and particularly that of the indole, if there is one, which is linked to the 4^i position. Accordingly, for the 4,4′-7′,4″-triindolyls **6** and **7**, all the H3^i resonate at high fields, each being indole linked through position 4, while in the case of **12** and **13** only the H3′ exhibit high field resonance frequency. Moreover, it must be noted how, for products **6–11**, the two atropoisomers present discrete variations in the chemical shifts of the shielded hydrogens H3 on the external units. This must be ascribed to the different spatial relative orientation of the first and the third indoles of the chain and the consequent variation in the shape of the ring current fields (Fig. 2). The H3 shielding is smaller when the two biindolyl subunits in the trimers have opposite configuration (*R*,*S* or *S*,*R*), as in **7**, **9**, **11**. This phenomenon is commonly observable in the analogous polynaphthyl linear chains as reported in literature[14] and further contribute to sustain the relative configuration derived on the basis of NOE effect. The observed shielding effects for H3 in **10**, **11** are higher than those of H3 in **6**, **7** probably because of proximity to the shielded hydrogen of a NH group instead of a CH group.[15]

Cooxidation of DHICA-**1b** and DHICA-**2b** presented the same behavior of the DHICA oxidation on dependence of oxidant systems. Moreover, the chromatographic profile of the **1b**-cooxidation mixture presented four main products which proved to be trimers **6–8**, **9**, corresponding to products presenting the highest yields among the trimers isolated from DHICA oxidation, while **2b**-cooxidation mixture presented the couples **6–7**, **10–11**, **12–13**.

By use of molecular modeling software (Hyperchem) geometry optimization of trimers was realized. This showed that, at the energetic minimum, the dihedral angle between contiguous indoles has an absolute value of about 47°12′ and the indoles of the chains have a conformational arrangement resembling that of polynaphthyl systems,[14b] each being a biindolyl subunit a chiral unit with *R* or *S* configuration. All the attempts to perform enantiomer separation by way of chemical derivatization with a chiral agent were discouraged because of the high reactivity of the dihydroxyindolyl chain, while enantiomer retention times, by chiral stationary phase chromatography, were not enough different to allow determination of optical activity.

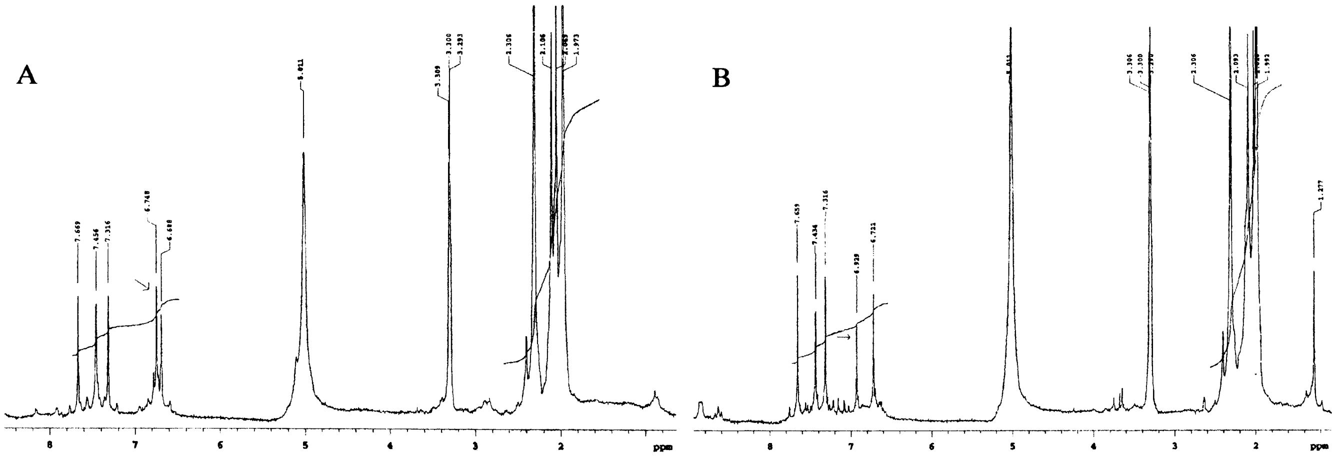

Figure 2. ^1H NMR spectra of **8** (A) and **9** (B) in CD_3OD taken with a Varian 200 MHz apparatus. Arrows indicate proton H3 which is deshielded in **9**.

3. Conclusions

Until now the knowledge of products arising from biomimetic DHICA oxidation was limited to a number of dimers and to a trimer which probably corresponds to **7**,[10] thus the isolation of products **6–13** represents a chief step forward in the elucidation of the structure of eumelanin polymers. Conclusive confirmation was also provided for the non-planar arrangement of contiguous indole units in DHICA oligomers because of steric hindrance also responsible for the atropoisomerism exhibited by these oligomers. The direct consequence of this structural feature is that each couple of contiguous indole units in the chain constitutes a chiral unit and thus atropoisomers may be regarded as diastereoisomers. This observation spurs new interest toward the photoprotective role of melanin pigments which could present some non-linear optical properties. Furthermore, there is no evidence for the existence of an extended conjugation of the indole units in such linear chains whose λ_{max} does not present significant differences from that of DHICA. In light of this, the black-brown color of eumelanin could not be ascribed to the absorbance features of the chromophoric oligomeric constituents, while it may derive from the effect of randomly disposed polarizing domains.

4. Experimental

4.1. General

For FAB-MS measurements, glycerol was used as matrix. ^1H 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 at 100.6 MHz using a Bruker XHCORR microprogram with a D_3 delay corresponding to J values of 140 and 10 Hz. Tetramethylsilane was used as 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 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 sulphate pentahydrate and all other chemicals were purchased from Aldrich. DHICA was synthetized by a literature procedure.[16] Glass distilled, deionised water was used for preparation of all solutions. Authentic samples of **1b** and **2b** were obtained as previously described.[11] Molecular geometry optimizations were carried out with Hyperchem 5.0 Package produced by Hypercube Inc. (Waterloo, Ont., Canada), 1997.

4.2. Oxidation of DHICA

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) sodium periodate (13 μmol) or (e) varying amounts of cupric sulphate (13, 26 or 52 μmol and 26, 52). In 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 **1b** and **2b** were identified by coinjection with authentic samples. Cooxidation DHICA-**1b** and -**2b** in 1:1 ratio was performed as above containing the starting solution 25 μmol of both DHICA and **1b**, **2b**; in case (d), cupric sulfate amounts were 19, 39 or 78 μmol.

4.3. Isolation of trimers 6–13

For the isolation of trimers **6–13**, a solution of DHICA (500 mg) in 0.1 M phosphate buffer, pH 7.0 (300 mL), was saturated with oxygen gas and then added with tyrosinase solution (12 mL, 400 units/mL) under vigorous stirring. After about 10 min, the mixture was reduced with an excess of sodium borohydride, acidified with HCl to pH about 2 and extracted three times with an equal volume of ethyl acetate. The organic layers were dried over sodium sulphate and evaporated to dryness. The residue (about 250 mg) was acetylated with acetic anhydride–pyridine 20:1 and chromatographed on preparative HPLC using as the mobile phase, 0.4 M formic acid–methanol: gradient from 7:3 to 1:1 over 90 min. The peaks eluted at ca. 20, 25, 35, 40, 47, 55, 60, 77 min were collected and carefully evaporated to dryness to give pure trimers **6** (40 mg), **7** (35 mg), **8** (15 mg), **9** (18 mg), **10** (8 mg), **12** (7 mg), **11** (9 mg), **13** (12 mg) in the order.

4.3.1. (4-4′*R*,7′-4″*R*)- and (4-4′*S*,7′-4″*S*)-5,5′,5″,6,6′,6″-Hexacetoxy-2,2′,2″-tricarboxy-4,4′:7′,4″-terindole (6). Pale gray to greenish vitreous solid. UV (MeOH): λ_{max} 304 nm. IR: ν_{max} (KBr)/cm^{-1} 3409, 1727, 1583, 1467, 750. FAB $(M+H)^+$: *m*/*z* 828; HRMS calculated for $C_{39}H_{30}N_3O_{18}$ (M^++H) 828.1524, found 828.1523. ^{1}H NMR (MeOH-d_4), δ (ppm): 1.94 and 1.95 (6H, bs, C5′- and C6′ –OCOCH$_3$), 2.07 (6H, bs, C5- and C5″–OCOCH$_3$), 2.31 and 2.30 (6H, s, C6- and C6″–OCOCH$_3$), 6.62 (1H, d, *J*=0.8 Hz, H3″), 6.66 (1H, d, *J*=0.8 Hz, H3), 6.69 (1H, s, H3′), 7.41 (1H, d, *J*=0.8 Hz, H7′), 7.45 (1H, d, *J*=0.8 Hz, H7″). ^{13}C NMR (MeOH-d_4), δ (ppm): 21.43 (2×OCOCH$_3$), 21.74 (2×OCOCH$_3$), 29.42 (2×OCOCH$_3$), 108.18 (C7), 108.91 (C7″), 109.49 (C3″), 110.12 (C3), 110.47 (C3′), 115.26 (C7′), 121.21 (C4″), 123.22 (C4 or C4′), 123.75 (C4 or C4′), 127.26, 127.29, 127.41 (C9, C9′ and C9″), 132.27, 132.56, 132.76 (C2, C2′ and C2″), 135.82 (C5′), 136.77 (C5 or C5″), 136.92 (C5 or C5″), 137.12 (C6′), 137.36 (C6 or C6″), 137.76 (C6 or C6″), 131.75 (C8′), 143.29 (C8 or C8″), 143.35 (C8 or C8″), 165.30, 165.44, 165.56 (3×COOH), 170.87, 171.13, 171.24, 171.34, 171.56, 171.79 (6×OCOCH$_3$).

4.3.2. (4-4′*R*,7′-4″*S*)- and (4-4′*S*,7′-4″*R*)-5,5′,5″,6,6′,6″-Hexacetoxy-2,2′,2″-tricarboxy-4,4′:7′,4″-terindole (7). Pale gray to greenish vitreous solid. UV (MeOH): λ_{max} 304 nm. IR: ν_{max} (KBr)/cm^{-1} 3410, 1720, 1583, 1466, 748. FAB $(M+H)^+$: *m*/*z* 828; HRMS calculated for $C_{39}H_{30}N_3O_{18}$ (M^++H) 828.1524, found 828.1522. ^{1}H NMR (MeOH-d_4), δ (ppm): 1.97 and 1.98 (6H, s C5′- and C6′–OCOCH$_3$), 2.10 (6H, bs, C5- and C5″–OCOCH$_3$), 2.31 and 2.32 (6H, bs, C6- and C6″–OCOCH$_3$), 6.70 (1H, s, H3′), 6.74 (1H, d, *J*=0.8 Hz, H3″), 6.80 (1H, d, *J*=0.8 Hz, H3), 7.42 (1H, d, *J*=0.8 Hz, H7), 7.46 (H1, d, *J*=0.8 Hz, H7″). ^{13}C NMR (MeOH-d_4), δ (ppm): 21.30 (2×OCOCH$_3$), 21.50 (2×OCOCH$_3$), 29.30 (2×OCOCH$_3$), 108.68 (C7), 109.21 (C7″), 109.88 (C3″), 110.50 (C3), 110.90 (C3′), 115.46 (C7′), 121.50 (C4″), 123.52 (C4 or C4′), 123.92 (C4 or C4′), 127.15, 127.25, 127.38 (C9, C9′ and C9″), 132.07, 132.34, 132.48 (C2, C2′ and C2″), 135.73 (C5′), 136.70 (C5 or C5″), 136.89 (C5 or C5″), 137.25 (C6′), 137.64 (C6 or C6″), 137.89 (C6 or C6″), 141.45 (C8′), 143.25 (C8 or C8″), 143.32 (C8 or C8″), 165.32, 165.41, 165.53 (3×COOH), 170.91, 171.22, 171.39, 171.43, 171.62, 171.96 (6×OCOCH$_3$).

4.3.3. (4-4′*R*,7′-7″*R*)- and (4-4′*S*,7′-7″*S*)-5,5′,5″,6,6′,6″-Hexacetoxy-2,2′,2″-tricarboxy-4,4′:7′,7″-terindole (8). Pale gray to greenish vitreous solid. UV (MeOH): λ_{max} 304 nm. IR: ν_{max} (KBr)/cm^{-1} 3412, 1724, 1584, 1466, 751. FAB $(M+H)^+$: *m*/*z* 828; HRMS calculated for $C_{39}H_{30}N_3O_{18}$ (M^++H) 828.1524, found 828.1528. ^{1}H NMR (MeOH-d_4), δ (ppm): 1.97 (6H, bs, C5′- and C6′–OCOCH$_3$), 2.05 and 2.11 (6H, bs, C5- and C6″–OCOCH$_3$), 2.31 (6H, bs, C5″- and C6–OCOCH$_3$), 6.69 (1H, s, H3′), 6.75 (1H, d, *J*=0.8 Hz, H3), 7.32 (1H, s, H3″), 7.46 (1H, d, *J*=0.8 Hz, H7), 7.67 (H1, s, H4″). ^{13}C NMR (MeOH-d_4), δ (ppm): 21.30, 21.35, 21.43, 21.59 (4×OCOCH$_3$), 25.01 (OCOCH$_3$), 31.53 (OCOCH$_3$), 108.97 (C7), 110.44 (2×C C3 and C3′), 111.13 (C3″), 114.20 (C7″), 114.92 (C7′), 118.34 (C4″), 122.96 (C4), 124.80 (C4′), 127.10, 127.40, 127.50 (C9, C9′ and C9″), 131.96, 132.31, 132.80 (C2, C2′ and C2″), 135.90 (C5 or C5′), 136.10 (C5 or C5′), 136.60 (C8′), 137.00 (C5″), 137.70 (C6), 139.29 (C8″), 141.50 (C6′ or C6″), 141.80 (C6′ or C6″), 143.29 (C8″), 165.24, 165.31, 165.40 (3×COOH), 171.28, 171.90, 172.09, 172.46, 172.68, 172.96 (6×OCOCH$_3$).

4.3.4. (4-4′*R*,7′-7″*S*)- and (4-4′*S*,7′-7″*R*)-5,5′,5″,6,6′,6″-Hexacetoxy-2,2′,2″-tricarboxy-4,4′:7′,7″-terindole (9). Pale gray to greenish vitreous solid. UV (MeOH): λ_{max} 304 nm. IR: ν_{max} (KBr)/cm^{-1} 3409, 1729, 1580, 1464, 750. FAB $(M+H)^+$: *m*/*z* 828; HRMS calculated for $C_{39}H_{30}N_3O_{18}$ (M^++H) 828.1524, found 828.1529. ^{1}H NMR (MeOH-d_4), δ (ppm): 1.99 (6H, bs, C5′- and C6′–OCOCH$_3$), 2.02 and 2.09 (6H, bs, C5- and C6″–OCOCH$_3$), 2.31 (6H, bs, C5″-e, C6–OCOCH$_3$), 6.72 (1H, s, H3′), 6.93 (1H, d, *J*=0.8 Hz, H3), 7.32 (1H, s, H3″), 7.43 (1H, d, *J*=0.8 Hz, H7), 7.66 (H1, s, H4″). ^{13}C NMR (MeOH-d_4), δ (ppm): 21.23, 21.38, 21.45, 21.61 (4×OCOCH$_3$), 25.03 (OCOCH$_3$), 31.33 (OCOCH$_3$), 108.87 (C7), 110.30 (2×C C3 and C3′), 111.53 (C3″), 114.21 (C7″), 115.00 (C7′), 118.03 (C4″), 122.26 (C4), 123.68 (C4′), 127.14, 127.54, 127.60 (C9, C9′ and C9″), 132.06, 132.21, 132.85 (C2, C2′ and C2″), 135.98 (C5 or C5′), 136.21 (C5 or C5′), 136.96 (C8′), 137.20 (C5″), 137.57 (C6), 139.59 (C8″), 141.76 (C6′ or C6″), 141.98 (C6′ or C6″), 143.49 (C8″), 165.22, 165.29, 165.34 (3×COOH), 171.32, 171.96, 172.11, 172.55, 172.78, 172.99 (6×OCOCH$_3$).

4.3.5. (4-7′*R*,4′-7″*R*)- and (4-7′*S*,4′-7″*SR*)-5,5′,5″,6,6′,6″-Hexacetoxy-2,2′,2″-tricarboxy-4,7′:4′,7″-terindole (10). Pale gray to greenish vitreous solid. UV (MeOH): λ_{max} 304 nm. IR: ν_{max} (KBr)/cm^{-1} 3410, 1726, 1584, 1464, 745. FAB $(M+H)^+$: *m*/*z* 828; HRMS calculated for $C_{39}H_{30}N_3O_{18}$ (M^++H) 828.1524, found 828.1519. ^{1}H NMR (MeOH-d_4), δ (ppm): 1.98 (6H, bs, C5′- and C6′–OCOCH$_3$), 2.06–2.12 (6H, bs, C5- and C6″–OCOCH$_3$), 2.30 and 2.32 (6H, bs, C5″- and C6–OCOCH$_3$), 6.65 (1H, d, *J*=0.8 Hz, H3), 6.58 (1H, s, H3′), 7.30 (1H, s, H3″), 7.49 (1H, d, *J*=0.8 Hz, H7), 7.63 (H1, s, H4″). ^{13}C NMR (MeOH-d_4), δ (ppm): 21.34, 21.46, 21.58, 21.79 (4×OCOCH$_3$), 24.89 (OCOCH$_3$), 31.52 (OCOCH$_3$), 109.26 (C7), 110.22 (2×C C3 and C3′), 111.82 (C3″), 113.48 (C7″), 115.76 (C7′), 117.65 (C4″), 119.94 (C4), 122.98 (C4′), 127.38, 127.54, 127.67 (C9, C9′ and C9″), 132.76, 132.83, 132.97 (C2, C2′ and C2″), 136.00 (C5 or C5′), 136.08 (C5 or C5′), 136.97 (C8′), 137.50 (C5″), 137.67 (C6), 140.83 (C8″), 140.96 (C6′ or C6″), 142.53 (C6′ or C6″), 143.38 (C8″), 165.32, 165.41, 165.54 (3×COOH), 171.78, 171.92, 172.18, 172.46, 172.54, 172.78 (6×OCOCH$_3$).

4.3.6. (4-7′*R*,4′-7″*S*)- and (4-7′*S*,4′-7″*R*)-5,5′,5″,6,6′,6″-Hexacetoxy-2,2′,2″-tricarboxy-4,7′:4′,7″-terindole (11). Pale gray to greenish vitreous solid. UV (MeOH): $\lambda_{\max}$ 304 nm. IR: $\nu_{\max}$ (KBr)/cm^{-1} 3409, 1724, 1580, 1467, 748. FAB (M+H)$^+$: *m*/*z* 828; HRMS calculated for $C_{39}H_{30}N_3O_{18}$ (M$^+$+H) 828.1524, found 828.1528. ^{1}H NMR (MeOH-*d*$_4$), δ (ppm): 2.00 (6H, bs, C5′- and C6′–OCOCH$_3$), 2.04 and 2.09 (6H, s, C5- and C6″–OCOCH$_3$), 2.29 and 2.33 (6H, s, C5″- and C6–OCOCH$_3$), 6.61 (1H, s, H3′), 6.90 (1H, d, *J*=0.8 Hz, H3), 7.30 (1H, s, H3″), 7.47 (1H, d, *J*=0.8 Hz, H7), 7.62 (H1, s, H4″). ^{13}C NMR (MeOH-*d*$_4$), δ (ppm): 21.48, 21.65, 21.80, 21.96 (4×OCOCH$_3$) 25.09 (OCOCH$_3$), 31.52 (OCOCH$_3$), 109.79 (C7), 110.69 (2×C C3 and C3′), 111.12 (C3″), 113.88 (C7″), 115.16 (C7′), 118.04 (C4″), 122.40 (C4), 123.58 (C4′), 127.21, 127.34, 127.48 (C9, C9′ and C9″), 132.45, 132.63, 132.87 (C2, C2′ and C2″), 135.99 (C5 or C5′), 136.03 (C5 or C5′), 136.83 (C8′), 137.38 (C5″), 137.78 (C6), 140.33 (C8″), 141.10 (C6′ or C6″), 142.25 (C6′ or C6″), 143.23 (C8″), 165.35, 165.48, 165.60 (3×COOH), 171.58, 171.89, 172.62, 172.76, 172.88, 172.99 (6×OCOCH$_3$).

4.3.7. (7-4′*R*,7′-7″*R*)- and (7-4′*S*,7′-7″*S*)-5,5′,5″,6,6′,6″-Hexacetoxy-2,2′,2″-tricarboxy-7,4′:7′,7″-terindole (12). Pale gray to greenish vitreous solid. UV (MeOH): $\lambda_{\max}$ 305 nm. IR: $\nu_{\max}$ (KBr)/cm^{-1} 3411, 1726, 1580, 1466, 751. FAB (M+H)$^+$: *m*/*z* 828; HRMS calculated for $C_{39}H_{30}N_3O_{18}$ (M$^+$+H) 828.1524, found 828.1524. ^{1}H NMR (MeOH-*d*$_4$), δ (ppm): 1.98 (6H, bs, C5′- and C6′–OCOCH$_3$), 2.11 (6H, bs, C6- and C6″–OCOCH$_3$), 2.30 (6H, bs, C5- and C5″–OCOCH$_3$), 6.62 (1H, s, H3′), 7.22 and 7.24 (2H, s, H3 and H3″), 7.61 (1H, s, H4″), 7.64 (H1, s, H4). ^{13}C NMR (MeOH-*d*$_4$), δ (ppm): 20.95, 21.28, 21.58, 21.90 (4×OCOCH$_3$), 25.07 (OCOCH$_3$), 31.51 (OCOCH$_3$), 110.69 (C3′), 111.12, 111.28 (2×C C3 and C3″), 112.79 (C7), 113.88 (C7″), 115.16 (C7′), 118.04 (C4), 120.40 (C4″), 124.88 (C4′), 127.71, 127.84, 127.98 (C9, C9′ and C9″), 132.25, 132.53, 132.67 (C2, C2′ and C2″), 135.79, 136.12, 136.30 (C5, C5′ and C5″), 136.87 (C8), 136.83 (C8′), 137.58, 138.10, 138.52 (C6, C6′, C6′), 140.33 (C8″), 166.01, 166.10, 166.20 (3×COOH) 171.28, 171.56, 172.02, 172.36, 172.48, 172.60 (6×OCOCH$_3$).

4.3.8. (7-4′*R*,7′-7″*S*)- and (7-4′*S*,7′-7″*R*)-5,5′,5″,6,6′,6″-Hexacetoxy-2,2′,2″-tricarboxy-7,4′:7′,7″-terindole (13). Pale gray to greenish vitreous solid. UV (MeOH): $\lambda_{\max}$ 304 nm. IR: $\nu_{\max}$ (KBr)/cm^{-1} 3405, 1726, 1580, 1466, 750. FAB (M+H)$^+$: *m*/*z* 828; HRMS calculated for $C_{39}H_{30}N_3O_{18}$ (M$^+$+H) 828.1524, found 828.1529. ^{1}H NMR (MeOH-*d*$_4$), δ (ppm): 2.00 (6H, bs, C5 and C5′–OCOCH$_3$), 2.04 and 2.09 (6H, s, C6- and C6″–COCH$_3$), 2.29 and 2.31 (6H, bs, C5′- and C6′–OCOCH$_3$), 6.66 (1H, s, H3′), 7.28 (2H, bs, H3 and H3″), 7.65 (1H, s, H4″), 7.68 (H1, s, H4). ^{13}C NMR (MeOH-*d*$_4$), δ (ppm): 21.58, 21.86, 21.89, 21.99 (4×OCOCH$_3$), 25.19 (OCOCH$_3$), 31.25 (OCOCH$_3$), 110.80 (C3′), 111.12, 111.28 (2×C C3 and C3″), 112.99 (C7), 113.86 (C7″), 115.20 (C7′), 118.14 (C4), 120.37 (C4″), 124.78 (C4′), 127.77, 127.82, 127.97 (C9, C9′ and C9″), 132.12, 132.56, 132.69 (C2, C2′ and C2″), 135.78, 136.15, 136.32 (C5, C5′ and C5″), 136.77 (C8), 136.84 (C8′), 137.50, 138.12, 138.42 (C6, C6′, C6′), 140.54 (C8″), 166.08, 166.13, 166.18 (3×COOH), 171.24, 171.45, 172.18, 172.23, 172.58, 172.62 (6×OCOCH$_3$).

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