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**Recommended protocols**

***Isolation of melanin from natural sources***

***Hair melanin*** *(Liu et al. 2005a, Novellino et al, 2000, Panzella et al 2007)*

Human hair (15 g) was homogenized in 200 ml of 0.1 M phosphate buffer, pH 7.5, with a glass pestle homogenizer and submitted to the following treatments: (1) dithiothreitol (3 g) was added to the homogenate, and the resulting mixture was taken under stirring at 37°C under a stream of argon for 18 h. Proteinase K (300 mg) and dithiothreitol (1.5 g) were then added to the mixture which was taken at 37°C under argon for additional 18 h. The mixture was centrifuged for 20 min (3,300 x g, 4°C). (2) The pellet was extensively rinsed with water and then suspended in 30 ml of 0.1 M phosphate buffer, pH 7.5, with papain (100 mg) and dithiothreitol (500 mg). The mixture was stirred for 18 h at 37°C under argon and centrifuged as above. (3) The black pellet collected, after 6-fold washing with water, was suspended in 30 ml of 0.1 M phosphate buffer, pH 7.5, added with protease (100 mg) and dithiothreitol (200 mg), and the mixture was stirred for 18 h at 37°C under an argon stream. (4) the dark residue obtained by centrifugation was suspended in 40 ml of 0.1 M phosphate buffer, pH 7.5, that had rigorously been purged with argon. An oxygen-free solution of 2 (w/v)% Triton X-100 was added up to 1 (w/v)% concentration, and the mixture was stirred for 4 h at room temperature under argon and then centrifuged for 20 min (106,000 x g, 4°C). After washing once with water: methanol, 1:1 (v/v), and four times with water, the black pellet was treated again with protease and dithiothreitol as described in (3). The final pigment pellet, collected by centrifugation, was dried over NaOH overnight and equilibrated with saturated aqueous CaCl2 for 24 h to give 720 mg of melanin.

***Iris melanin*** *(Liu et al. 2005b, Novellino et al, 2000)*

Bovine irides (11 g wet tissue) were homogenized in 200 ml of 0.1 M phosphate buffer, pH 6.8, with a glass pestle homogenizer and submitted to the following treatments: (1) collagenase (68 U/ml) and CaCl2 (2 mM) were added to the homogenate, and the incubation mixture was stirred at 37°C under a stream of argon for 16 h and then centrifuged for 25 min at 3,300 x g at 4°C. (2) The dark pellet thus obtained was washed five times with water and then suspended in 50 ml of 0.05 M phosphate buffer, pH 7.5, in the presence of pancreatin (125 mg). The mixture was stirred for 2 h at 37°C under argon and centrifuged as above. After two-fold washing with water, the pigmented residue was suspended in 50 ml of 0.05 M phosphate buffer, pH 7.5, trypsin (32,300 U) and CaCl2 (2 mM) were added, and the mixture was stirred for 2 h at 37°C under a stream of argon. The black pellet was collected by centrifugation and washed as above. (3) The material was suspended in 40 ml of 0.1 M phosphate buffer, pH 6.8, that had rigorously been deaerated with argon, and then an oxygen-free solution of 2 (w/v)% Triton X-100 in 0.1 M phosphate buffer, pH 6.8, was added up to 1 (w/v)% final concentration. After stirring for 1.5 h at room temperature under argon, the mixture was centrifuged for 30 min at 106,000 x g The black pellet was washed once with water: methanol, 1:1 (v/v), and four times with water, and then treated again with collagenase as described in (1). After centrifugation and washing with water, the recovered material was dried over NaOH overnight and equilibrated with CaCl2 for 24 h to afford 190 mg of melanin.

***B16 mouse melanoma*** *(Palumbo et al, 1994)*

B16 melanoma tissue was homogenized in ethanol/1% acetic acid (70:30 v/v) centrifuged at 17,300 x g for 15 min and the resulting precipitate was washed three times with water. The washed melanin was then further purified by sucrose-density-gradient (30 %, 25 %, 20 % and 15 %) ultracentrifugation at 150,000 x g for 60 min. The melanosomal fraction obtained at the bottom of 30 % sucrose was washed with water and treated three times with 1% Triton X-100/0.1 % SDS in 0.1 M-Tris/HCI, pH 7, overnight at 23°C. The melanin pellet was then again extensively washed with water, dried in a silica-gel desiccator, and equilibrated in the presence of saturated CaCl2 until a constant weight was obtained.

**Neuromelanin (NM) from substantia nigra (SN)**

NM can be isolated from the SN *pars compacta* region of the human midbrain by dissecting the SN from the frozen human midbrain on a cold plate at −10°C within 36 h of death followed by homogenization in distilled H2O (0.03 g/ml) in a glass-Teflon homogenizer and centrifugation at 12,000 g for 10 min. The resulting pellet was washed twice with 50 mM phosphate buffer (pH 7.4), incubated in 50 mM Tris buffer (pH 7.4) containing 0.5 mg/ml SDS at 37°C for 3 h, and incubated for another 3 h at 37°C in the same buffer containing 0.2 mg/ml proteinase K. The resulting pellet was washed twice with NaCl (9 mg/ml), three times with distilled water, and once each with methanol (2 ml) and hexane (1 ml). Finally, it was dried under vacuum and stored protected from light. The procedure described herein is the same as used in a previous study on the characterization of pheomelanin and eumelanin content in NM. Reproducibility of pheomelanin and eumelanin composition for the granules studies herein was confirmed by elemental analysis of carbon, hydrogen, nitrogen, sulfur, and oxygen.

**Neuromelanin from pc12 cells**

For purification of NM from cells, frozen cells (typically 3 x 108) were thawed at room temperature, washed in 10 ml of 0.05 N phosphate buffer pH 7.2, centrifuged at 10,000 x g for 15 min at room temperature , washed, and re-centrifuged. Samples received 10 ml of 5 mg/ml SDS in 75 mM Tris, pH 7.5, were sonicated, and incubated for 3 h at 37°C in a shaking water bath. The preparations were centrifuged at 10,000 x g at room temperature for 30 min and the supernatant removed. Two ml of 5 mg/ml SDS in 75 mM Tris, pH 7.5 with 0.33 mg/ml proteinase K (Sigma) was added to the pellet and incubated for 3 h at 37°C. The samples were centrifuged at 10,000 x g at room temperature for 30 min and the supernatant removed. The pellets were washed with 3 ml 0.9% NaCl and centrifuged at 10,000 x g at room temperature, the supernatant discarded, and the pellet transferred to corex tubes. The pellet was washed with 3 ml of water, and centrifuged at 10,000 x g at room temperature for 30 min and the supernatant removed. The pellet was rewashed in 3 ml of methanol followed by 3 ml of hexane and centrifuged at 10,000 x g at room temperature for 30 minutes. Isolated NM was dried under N2 gas and stored at -80°C.

**Preparation of melanin precursors and degradation products**

**1) DHI and DHICA**

The methods are essentially similar to those reported (Wakamatsu and Ito, 1988, Edge et al, 2006) with minor modifications. A solution of 985 mg (5 mmol) dl-dopa in water (500 ml) and a solution of K3[Fe(CN)6] (6.6 g, 20 mmol) and NaHCO3 (2.5 g, 30 mmol) in water (60 ml) were separately degassed by aspirating with a water aspirator for 10 min or purging with an argon flux for 10 min. The solution of K3[Fe(CN)6] and NaHCO3 was poured at once to the solution of dopa while stirring at room temperature. For the preparation of DHI, the wine-red solution of dopachrome was stirred and degassed (aspirating or purging) at room temperature for 2 h during which CO2 evolution gradually ceased. Solid Na2S2O5 (5.0 g, 26 mmol) was then added to the dark brown solution, which was extracted with ethyl acetate (300 ml x 3). The ethyl acetate extracts were combined, washed once with saturated NaCl solution (100 ml), and dried over anhydrous Na2SO4 (50 g). Evaporation of ethyl acetate gave a brown oil, which was dissolved in acetone (3 ml) plus benzene (30 ml), and to the solution hexane (20 ml) was added. Black melanin precipitate was removed by filtration, and the resulting brown solution was gradually mixed with hexane (100 ml) to give almost colorless crystals of DHI. The mixture was kept overnight at 4˚C and the crystals were filtered and dried to give 451 mg (61% yield) of DHI. Several preparations gave yields of 61-64% (in the literature, the yield was 40%). HPLC analysis revealed no contamination of DHICA. Elemental analysis: calculated for C8H7NO2: C 64.42%, H 4.73%, N 9.39%; found: C 64.40%, H 4.69%, N 9.31%. UV (EtOH) max 275 nm (6,520) and 303 nm (9,710); 1H and 13 C NMR spectrum in fig 1.For the preparation of DHICA, the wine-red solution of dopachrome was stirred for 2 min (critical!) and mixed with 1 M NaOH (70 ml), which had been degassed by aspirating with a water aspirator for 10 min or purging with an argon flux for 10 min. After 15 min at room temperature, solid Na2S2O5 (5.0 g, 26 mmol) and then 6 M HCl (19 ml) were added to the dark brown solution. The resulting pale brown solution was extracted with ethyl acetate (300 ml x 3). The extracts were combined, washed once with saturated NaCl solution (100 ml), and dried over anhydrous Na2SO4 (50 g). Evaporation of ethyl acetate gave a purple crystalline powder, which was dissolved in acetone (20 ml), and hexane (30 ml) was added. Dark brown melanin precipitate was removed by filtration, and the resulting brown solution was gradually mixed with hexane (100 ml) to give almost colorless crystals of DHICA. The mixture was kept overnight at 4˚C and the crystals were filtered and dried to give 718 mg (74% yield) of DHICA. Several preparations gave yields of 74-80% (in the literature, the yield was 38%). HPLC analysis revealed no contamination of DHI. Elemental analysis: calculated for C9H7NO4: C 55.96%, H 3.65%, N 7.25%; found: C 55.75%, H 3.88%, N 6.98%. UV (EtOH) max 321 nm ( 18,450); 1H and 13 C NMR spectrum in fig S2.

**2) Preparation of 5SCD**

**Experimental procedure a**

*Reaction :* To a solution of l-dopa (1.97 g, 10 mmol) in 2 M sulphuric acid (50 ml) a solution of cerium ammonium nitrate (10.96 g, 20 mmol) in 2 M sulphuric acid (100 ml) is added under vigorous stirring at room temperature. After ca 3 min the deep red solution is poured in a solution of l-cysteine (5.02 g, 41 mmol) in 2 M sulphuric acid (50 ml) under a flux of argon. After five min the color of the solution turns to blue-green. HPLC elution profile of the reaction mixtureusing a wettable Hydro C-18 column (0.46 cm x 25 cm) flow rate 0.7 ml/min, eluant 0.2% trifluoroacetic acid/acetonitrile 95:5 is shown in Fig S3 top panels with detection at 280 and 254 nm. Formation yield of 5SCD is estimated as much as 75%.

*Critical points: i) cerium ammonium nitrate is somewhat hygroscopic and it should be extensively dried under vacuum before use; ii) addition of the dopaquinone solution to cysteine solution should be as rapid as possible. Contact with air of the final mixture should be avoided or reduced to a minimum.*

*Fractionation Step 1.* The reaction mixture is loaded on a cationic exchange resin (Dowex 50W-X8, 200-400 mesh, H+ form) column (2.5 cm x 60 cm). Elution is carried out with water (0.5 L), 0.5 M HCl (4 L) and 3 M HCl (5 L). Fractions eluted with 3M HCl are reduced to small volume (2 ml) under vacuum.

*Critical points: column elution should be monitored by UV visible spectrophotometry. Only the eluates from 3 M HCl should be better collected in small fractions (20-25 ml) and analyzed individually by UV spectrophotometry. Fractions exhibiting absorbances at 292 and 253 are collected and subjected to further purification. HPLC analysis of the latter fractions under the conditions indicated above is shown below (fig S3 bottom panels).*

*Fractionation Step 2. Method A* The mixture obtained from step 1 is reduced to small volume (10 ml) and is further purified by preparative HPLC using a Econosil C-18 column (2.2 cm x 25 cm) flow rate 20 ml/min, eluant 0.2% trifluoroacetic acid /acetonitrile 95:5. Under these conditions 5SCD is eluted at around 8 min, while the other isomers are less retained and elute as an unresolved peak centred at 4.6 min. The eluates are reduced to dryness, taken up in 0.1M HCl (3 x 30 ml). After removal of the solvent, the residue is taken up in methanol (10 ml) and the solution is slowly added to ethyl acetate (500 ml). The solid which separates is collected by centrifugation (5,000 rpm, 30 min, 20°C) and dried under vacuum. The yield of 5SCD ranges is around 60%.

*Critical points: Typically, 20-25 runs with injection of 0.5 ml of mixture are required for purification of a mixture obtained from 10 mmol of dopa.*

*Fractionation Step 2. Method B* The mixture obtained from step 1 is reduced to small volume (ca 2 ml) and loaded on a Dowex 50W-X8 (200-400 mesh, H+ form) column (2.0 x 40 cm) equilibrated with 2 M HCl and eluted with 2M HCl collecting 15 mL-fractions. Fractions containing 5SCD as determined by UV-vis spectrophotometry analysis are taken to dryness to give the product as HCl salt. This may be obtained as solid by crystallization. The residue is taken up in water and the solution is added with Na2S2O5 (1%). Dropwise addition of saturated sodium acetate leads to separation of 5SCD as the free base in 50 to 60% yields.

*Critical points: elution of 5SCD should be followed by careful examination of the absorption spectrum of the collected fractions. Only those showing a 1:1.25 ratio of the 254 and 293 nm abs max are collected together.*

**Cautions and comments.**

Sulphuric acid is highly caustic, dilution of conc. sulphuric acid by addition to water results in a sudden warming. Dopaquinone is highly unstable even in acid and to avoid its degradation it should be allowed to react with cysteine very rapidly. It is advisable to keep the reaction mixture under controlled atmosphere to avoid further oxidation of cysteinyldopas as formed by oxygen. This can not be completely avoided as indicated by the blue color of the final mixture due to small amounts of trichochrome type oxidation products. The first purification step removes residual DOPA and cysteine, as well as saline components including the oxidant affording a mixture of cysteinyldopa isomers.

Purification of 5SCD from isomers may be obtained by reverse phase chromatography using a preparative HPLC column. Given the availability of the instrument this method is not very expensive or time consuming as, under the conditions developed, elution is very rapid and the mobile phase contains small amounts of organic phase, so the eluant that is required in large volumes is low cost.

**Experimental procedure b**

Enzymatic method: The method was essentially similar to that reported in Ito and Prota (1977), but the scale was increased 4-fold. A solution of 394 mg (2 mmol) l-dopa and 484 mg (4 mmol) l-cysteine in 0.1 M sodium phosphate buffer, pH 6.8 (195 ml), was vigorously stirred at 25˚C in a 1 LErlenmeyer flask. To this solution a solution of 37.2 mg (200,000 U) mushroom tyrosinase (5,370 U/mg; from Sigma-Aldrich) in the sodium phosphate buffer (5 ml) was added in one portion, and the mixture was vigorously stirred. After 30 min, HPLC and UV spectral analysis showed that the remaining dopa was ca. 10% and the yields of 5SCD and 2SCD reached a plateau, and the mixture was poured onto a Dowex 50W-X2 (200-400 mesh) column (3.3 cm x 15 cm, H+ form, equilibrated with water). After washing with 0.5 M HCl (500 ml), the column was eluted with 3 M HCl and fractions of 100 ml were collected and analyzed by HPLC and UV spectrophotometry (diluted with 0.1 M HCl). Fractions 3-9 containing cysteinyldopas including 2,5-*S,S*-dicysteinyldopa (DiCD) were combined and evaporated to dryness. A glassy residue was taken up in 2 M HCl (6 ml) and chromatographed on a Dowex 50W-X2 (200-400 mesh) column (3.3 cm x 24 cm, equilibrated and eluted with 2 M HCl). Fractions of 100 ml (22 fractions) were collected (100 ml/h) and analyzed by HPLC and UV spectrometry. Fractions 11-14 and 15-22 were found to contain 5SCD and 2SCD, respectively. (Further elution with 4 M HCl yielded 25 mg of DiCD from fr. 24-27.) Combined fractions were evaporated to dryness to give 2HCl salts of 5SCD (611 mg) and 2SCD (103 mg), which were dissolved in 2% Na2S2O5 (8 ml and 2 ml, respectively). The solutions were neutralized to pH ca. 5 with addition of saturated sodium acetate solution while stirring. After keeping overnight at 4˚C, colorless crystals of 5SCD and 2SCD were obtained, which were collected, washed with water, and dried. 5SCD and 2SCD were obtained in 440 mg (66% yield) and 56.0 mg (8.4% yield), respectively. HPLC analysis revealed isomeric purity of 99%. 5SCD, elemental analysis: calculated for C12H16N2O6S.H2O: C 43.10, H 5.53%, N 8.38%, S, 9.59%; found: C 42.86%, H 5.16%, N 8.23%, S 9.45; UV (0.1M HCl): max 254 nm ( 3,600) and 293 nm (2,900); 1H and 13 C NMR spectrum in fig S4 2SCD, elemental analysis: calculated for C12H16N2O6S.H2O: C 43.10, H 5.53%, N 8.38%, S, 9.59%; found: C 42.51%, H 5.13%, N 8.21%, S 9.32%. UV (0.1M HCl): max 255 nm ( 2,280) and 294 nm (3,030) 1H and 13 C NMR spectrum in fig S5 .

**Comments.** This method requires only two days for the oxidation and the two chromatography steps. The yield of CDs depends on the amount (unit) of mushroom tyrosinase. With 250,000 units of mushroom tyrosinase, no dopa remained, but the yield of 5SCD was reduced a little with a 3.5-fold greater yield of DiCD. This is because 5SCD, as a substrate of tyrosinase, is slowly converted to DiCD when all the dopa is consumed (Agrup et al., 1982).

**3) PTCA and PDCA**

The methods follow those previously reported (Wakamatsu and Ito, 1998) but are much improved in yields. For the preparation of PTCA, a solution of 708 mg (4 mmol) of 5-hydroxyindole-2-carboxylic acid in 1 M K2CO3 (100 ml) was mixed with 30% H2O2 (6 ml, 60 mmol) and stirred at room temperature for 24 h. After addition of 10% Na2SO3 (20 ml), the mixture was acidified to pH 1 with 6 M HCl (40 ml) and extracted with diethyl ether (200 ml x 3). The combined extracts were washed once with saturated NaCl solution (50 ml), and dried over anhydrous Na2SO4 (40 g). Evaporation of ether gave a brown crystalline powder, which was dissolved in acetone (6 ml), and the solution was gradually mixed with hexane (100 ml) to give pale yellow crystals of PTCA. The mixture was kept overnight at 4˚C and the crystals were filtered and dried to give 285 mg (36% yield) of PTCA. Purity >99% by HPLC. Elemental analysis: calculated for C7H5NO6: C 42.22%, H 2.53%, N 7.04%; found: C 42.51%, H 2.72%, N 6.75%. UV (0.1 M HCl ): max 230 nm ( 23,510) and 272 nm (12,940) ; 1H and 13 C NMR spectrum in fig S6.

For the preparation of PDCA, a solution of 532 mg (4 mmol) of 5-hydroxyindole in 1 M K2CO3 (100 ml) was mixed with 30% H2O2 (6 ml, 60 mmol) and stirred at room temperature for 24 h. After addition of 10% Na2SO3 (20 ml), the mixture was acidified to pH 1 with 6 M HCl (40 ml) and extracted with diethyl ether (200 ml x 3). The combined extracts were washed once with saturated NaCl solution (50 ml), and dried over anhydrous Na2SO4 (40 g). Evaporation of ether gave a brown crystalline powder, which was dissolved in acetone (6 ml), and the solution was gradually mixed with hexane (60 ml) to give pale pink crystals of PDCA (the first oily precipitate should be discarded). The mixture was kept overnight at 4˚C and the crystals were collected and dried to give 112 mg (17% yield) of PDCA. Purity >99% by HPLC. Elemental analysis: calculated for C6H5NO4.0.5H2O C 43.91%, H 3.69%, N 8.53%; found: C 43.87%, H 3.55%, N 8.61%. UV ( 0.1 M HCl)max 246 nm ( 6,700) and 288 nm (9,440) ; 1H and 13 C NMR spectrum in fig S7.**4) 4-AHP**

HI hydrolysis of synthetic 5SCD-melanin afforded 4-AHP in 12% yield (Wakamatsu et al., 2009). However, this method requires preparation of 5SCD and its oxidation to melanin, which are tedious. On the other hand, the reported multi-step chemical synthesis starting from 3-hydroxybenzaldehyde appeared rather complex (Fattorusso et al, 1968). Thus, an alternative chemical method was newly developed starting from commercially available dl-*m*-tyrosine.

A suspension of 500 mg (2.76 mmol) dl-*m*-tyrosine was mixed with 60% HNO3 (1.0 ml, 12.1 mmol). The resulting solution was stirred at room temperature for 60 min, leading eventually to a strongly dark red solution. HPLC analysis confirmed the absence of *m*-tyrosine and the presence of 3 major products corresponding most likely to nitro-*m*-tyrosines resulting from nitration at the *o-* and *p-*positions of the hydroxyl group. The mixture was diluted with water (10 ml) and applied onto a Dowex 50W-X2 (200-400 mesh) column (1.5 cm x 6 cm, H+ form, equilibrated with water). The column was washed with water (100 ml) and then the products were eluted with 2 M HCl (20 ml/fractions). Fractions 2-8 containing nitrated *m*-tyrosine isomers were combined and evaporated to dryness to give 545 mg (75%) of a dark brown powder.

The mixture of nitrated *m-*tyrosines was then heated under reflux with 57% HI (10 ml) in the presence of 50% H3PO2 (1.0 ml). After 60 min, HPLC analysis confirmed the absence of the nitrated *m*-tyrosines and the appearance of 3 major products corresponding most likely to 4-amino-3-hydroxyphenylalanine (4A3HP; 4-AHP), 2-amino-5-hydroxyphenylalanine (2A5HP), and 2-amino-3-hydroxyphenylalanine (2A3HP). The mixture was evaporated to dryness, taken up in 0.1 M HCl (3 ml), and chromatographed on a Dowex 50W-X2 (200-400 mesh) column (1.8 cm x 31 cm, equilibrated and eluted with 2 M HCl). Fractions of 20 ml were collected and analyzed by UV spectrophotometry and HPLC (ODS column, 0.1 M potassium phosphate buffer, pH 2.1: methanol = 99:1, 45˚C, 0.7 ml/min). 4-AHP (fr 12-18) was well separated from the other isomers 2A5HP (major; fr. 20-29) and 2A3HP (minor; fr 22-30). Evaporation of fr 12-18 and fr 20-30 gave 236 mg and 229 mg of crystalline 2HCl salt of the AHP isomers, respectively. Recrystallization of 4-AHP (fr. 12-18) from 6 M HCl (3 ml) plus acetone (60 ml) gave colorless crystals of 4-AHP. 2HCl in 220 mg (23% yield). Purity 99% by HPLC. Elemental analysis: calculated for C9H12N2O3.2HCl. CH3COCH3 C 44.05%, H 6.16%, N 8.56%, Cl 21.67%; found: C 44.77%, H 5.98%, N 8.49%, Cl 20.47%. UV (0.1M HCl) λmax215 nm ( 6,840) and 275 nm (2,370) ; 1H and 13 C NMR spectrum in fig S8.

**5) 4-AHPEA and 3-AHPEA**

Authentic samples of 4-AHPEA and 3-AHPEA can be prepared by HI reductive hydrolysis of 5-*S*-cysteinyldopamine (CysDA)-melanin and 2-*S*-Cys-DA-melanin, respectively (Wakamatsu et al, 2003). 5-*S*-CysDA-melanin and 2-*S*-Cys-DA-melanin were synthesized by tyrosinase oxidation of 5-*S*-CysDA and 2-*S*-CysDA prepared by the reported method (Ito *et al*, 1986). It should be noted that CysDA-melanin is insoluble in neutral buffer but is soluble at acidic pH thus acidification of the melanin preparation mixtures must be avoided. The yields of 4-AHPEA and 3-AHPEA were 12% and 12%, respectively. UV (0.1 M HCl) max 276 nm ( 1,700) for 3-AHPEA; 1H and 13C NMR in fig. S9. UV (0.1 M HCl) max 274 nm ( 1,910) for 4-AHPEA, 1H and 13C NMR in fig. S10. Alternative methods based on nitration of *p*- and *m*-tyramines followed by reductive amination (with HI) of the nitro-tyramines are now being developed and will be reported elsewhere.

**6) BTCA/BTCA-2 and BT/BT2**

**a) from dopa and cysteine**

A solution of l-dopa (158 mg, 0.80 mmol) in 0.05 M phosphate buffer, pH 7.4 (34 ml) was sequentially treated with l-cysteine (194 mg, 1.60 mmol) and mushroom tyrosinase (77,800 units) and the mixture was taken under vigorous stirring at room temperature. After 2 h the mixture was treated with a solution of zinc sulfate heptahydrate (275 mg, 0.96 mmol) in water (4 ml) and a solution of K3[Fe(CN)6] (260 mg, 0.80 mmol) in 0.05 M phosphate buffer, pH 7.4 (4 ml); after 20 min, Na2S2O8 (570 mg, 2.40 mmol) and 12 M HCl (40 ml) was added to the oxidation mixture and, after additional 20 min, the reaction mixture was treated with NaHSO3 (305 mg, 1.60 mmol). The resulting mixture was fractionated by preparative HPLC (10 **m particle size 250 × 22 mm Econosil ODS, 0.2% trifluoracetic acid/methanol 65:35, 20 ml/min). After evaporation of the solvent the residue was dissolved in 0.1 M HCl and taken to dryness to afford BTCA(Napolitano *et al*, 1996) as hydrochloride salt (145 mg, 57% yield). The oxidation mixture obtained as above (Greco *et al*, 2009a) was heated at 90 °C for 1.5 h. The fraction obtained by preparative HPLC (column and solvent rate as above, eluent trifluoroacetic acid/methanol 75:25) was taken to dryness and treated with 0.1 M HCl to afford BT (Di Donato *et al*, 2002) as hydrochloride salt (120 mg, 55% yield).

**b) from cysteinyldopa isomers**

The reaction of 5SCD or 2SCD (250 mg, 0.80 mmol) was carried out in 0.05 M phosphate buffer (pH 7.4) (34 mL) using ZnSO4, K3[Fe(CN)6] and Na2S2O8 at the same reagent/substrate ratios indicated above followed by preparative HPLC. The residue was dissolved in 0.1 M HCl and taken to dryness to afford BTCA(152 mg, 60% yield). UV (0.5 M HCl) max 250 nm ( 7,600), 289 nm (4,400), and 332 nm (1,550); ESI (+) MS *m*/*z* 283 [M+H]+ or BTCA-2 (145 mg, 57% yield). UV (0.5 M HCl) max 252 nm ( 7,600), 287 nm (4,300), and 346 nm (1,300); ESI(+) MS *m*/*z* 283 [M+H]+ (Greco et al 2009b) respectively,as hydrochloride salt. The mixture obtained by oxidation of 55SCD or 2SCD was heated at 90 °C for 1.5 h, and purified by preparative HPLC under the conditions described above. Treatment of the residue with 0.1 M HCl afforded BT(125 mg, 57% yield, 1H and 13C NMR spectra in fig S11) or BT2 (Patil and Chedekel, 1984) (114 mg, 52% yield, 1H and 13C NMR spectra in fig S12) respectively, as hydrochloride salt.

**6) TTCA and TDCA**

TTCA preparation followed a method reported in the literature (Erlenmeyer et al, 1948). A mixture of 1.20 g of ethyl thiooxamate (9.02 mmol) and 1.75 ml of diethyl chlorooxaloacetate (9.02 mmol) was stirred at 60˚C for 2 h. Yellow precipitates were generated. After cooling, the reaction mixture was added with 3 ml saturated sodium acetate solution and extracted with ether (30 ml x 3). The combined ether extract was washed with saturated NaCl and dried over anhydrous Na2SO4. Evaporation of ether gave crude yellow oil: 2.20 g pale yellow oil of triethyl thiazole-2,3,5-tricarboxylate (81% yield) was obtained after purification by silica gel column chromatography (12 g, hexane : ethyl acetate = 2 : 1).

A solution of 1.10 g (3.65 mmol) triethyl thiazole-2,3,5-tricarboxylate in ethanol (2 ml) was mixed with KOH (0.74 g, 13.1 mmol) in water (2 ml) and the mixture was stirred at room temperature for 1 h. After generation of heat, pale yellow precipitate was obtained. The precipitate of tripotassium salt was filtrated and washed with ethanol. The precipitate was redisssolved with water (1 ml) and recrystallized by adding ethanol (15 ml). 1.21 g (93% yield) tripotassium salt of TTCA was obtained as colorless crystals. Purity > 99% by HPLC. Elemental analysis: calculated for C6NO6SK3.1.5H2O C 20.10%, H 0.84%, N 3.91%, S 8.95%; found: C 20.93%, H 0.98%, N 3.51%, S 8.40%. UV (0.1 M HCl) λmax 217 nm(ε25,350) and 275 nm(12,030): ES(+) MS (332 [M3K+H] + 13C NMR spectrum in fig S13.

For the preparation of TDCA, we took advantage of the facile decarboxylation of TTCA (Erlenmyer et al, 1948). A solution of 331 mg (1 mmol) of tripotassium TTCA in 0.5 M HCl (10 ml) was heated for 10 min in which decarboxylation completed. The mixture was evaporated in vacuo, and the residue, dissolved in water (5 ml), was chromatographed on Dowex 50W-X2 (200-400 mesh) column (1.5 cm x 6 cm, H+ form, equilibrated with water). Fractions of 10 ml were collected and analyzed by UV spectrophotometry. Fractions 2 and 3 were found to contain most of TDCA (actually TDCA became crystals in the fraction 2) and were evaporated to dryness, giving 176 mg of colorless crystals of TDCA. Recrystallization from water (3 ml) + 6 M HCl (0.1 ml) afforded 142 mg (82% yield) of TDCA. Purity >99% by HPLC. Elemental analysis: calculated for C5H3NO4S C 34.68%, H 1.75%, N, 8.09%, S 18.52%; found: C 34.38%, H 1.79%, N 8.16%, S, 18.42%. UV (0.1 M HCl) λmax 206 nm (e 10,950) and 254 nm (6,440). ES (+) MS 174 [M+H]+, 196 [M+Na]+ ; 1H and 13 C NMR in fig S14.

**Preparation of synthetic eumelanin and pheomelanin**

**Procedure a : tyrosinase**

As precursor(s) of melanin, 1) l-dopa, 2) DHI, 3) a mixture of DHI and DHICA, 4) DHICA, 5) a mixture of l-dopa and l-cysteine, and 6) 5SCD in the presence of l-dopa was used. The amount of mushroom tyrosinase (Sigma-Aldrich, 4270 U/mg) was either 50,000 U or 100,000 U (see Table 1 in the main text). To a solution of the precursor(s) (1 mmol) in 0.1 M sodium phosphate buffer, pH 6.8 (98 ml) a solution of mushroom tyrosinase in the same buffer (2 ml) was added at 25˚C. The oxidation was carried out with vigorous (maximal, but avoiding splashing) stirring in a 500 ml Erlenmeyer flask. Oxidation (1-4) was stopped at 4 h by adding 6 M HCl (2 ml) to make pH 1 and the mixture was kept at 4˚C overnight to ensure complete precipitation of the pigment. The precipitate was collected by centrifugation at 3,000 rpm for 10 min and washed with 0.1 M HCl (40 ml x 3). For the preparation of pheomelanins (5 and 6), the oxidation mixture was acidified with acetic acid (3 ml) to make pH 3, and washed the precipitate with 1% acetic acid (40 ml x 3). The melanin powder was dried by lyophilization and equilibrated with moisture in a desiccator with a saturated CaCl2 solution. Elemental analysis and characterization of the melanin pigments are summarized in Table 1.

**Procedure b : peroxidase/H2O2/(Zinc ions)**

As precursor(s) of melanin, 1) DHI, 2) a mixture of DHI and DHICA, 3) DHICA, (Napolitano et al 1995) 4) 5SCD and 5) 2SCD. (Napolitano et al, 2008, Panzella et al 2010) were used. The appropriate substrate (1 mmol) was dissolved in 0.1 M phosphate buffer, pH 6.8 (80 ml) and treated sequentially with horseradish peroxidase (1,300 pyrogallol units) and 30% hydrogen peroxide (120 l). After 2 h under stirring at 25°C, the mixture was acidified to pH 3 and the pigment formed was collected by centrifugation at 7,500 rpm for 15 min and then washed three times with 0.1 M HCl. For preparation of pheomelanin from cysteinyldopas, the melanin precipitate was collected by centrifugation at 4°C, at 7,500 rpm for 15 min and washed three times with 1% acetic acid, once with water. For preparation of pheomelanin pigments in the presence of zinc, ZnSO4.7H2O (1.2 molar equivalents) was added to the solution of the substrate followed by peroxidase (300 pyrogallol units/ml) and hydrogen peroxide (680 l) added in two portions at 5 h intervals. The mixture was taken under stirring for 24 h and then acidified to pH 3, and the melanin precipitate was collected as above. In all cases the pigments are dried by lyophilization.

**Quantitative determination of melanin by EPR**

To increase the sensitivity of EPR determination of melanin in fully hydrated samples, measurements are carried out at low temperature, typically at 77K in liquid nitrogen (Sarna et al., 2003). For quantitative determination of melanin, standard X-band EPR spectroscopy operating at about 9 GHz microwave frequency is the best choice. EPR samples are prepared in the following way: aqueous suspension of the examined material, after transfer to thick-wall glass cylinders (~4 mm I.D, ~ 3 cm long), is frozen by carefully submerging one of its ends sealed with parafilm in liquid nitrogen. The formed icicle is then removed from the cylinder and transferred to a quartz finger tip dewar by gentle warming of the cylinder surface. To optimize EPR measurements, the length of the frozen sample should be 2-2.5 cm. To minimize noise due to liquid nitrogen bobbling and the icicle movement during EPR determination, the icicle should be immobilized with the use of a quartz rod and the icicle thickness should be close to that of the inner diameter of the dewar finger-tip. The amount of melanin in the samples studied is determined relative to an appropriate standard run under identical experimental and apparatus conditions. Frozen suspension of known amounts of natural or synthetic melanin is a reasonable standard. Apparatus settings should be carefully chosen to avoid microwave power saturation of the melanin EPR signal and its excessive broadening due to magnetic overmodulation. Estimation of melanin concentration is based on comparison of double integrals of the melanin EPR signals in the examined samples and that of the standard. If the signal-to-noise ratio is not particularly good, better estimates of the melanin can be obtained by comparing the corresponding products H)2 of the EPR signals of the sample and standard, where A is the signal amplitude and H is the signal linewidth.

**References**

# Agrup, G., Hansson, C., Rorsman, H., and Rosengren, E. (1982). The effect of cysteine on oxidation of tyrosine, dopa, and cysteinyldopas. Arch. Dermatol. Res. *272*, 103-115

Di Donato, P., Napolitano, A., and Prota, G. (2002).Metal ions as potential regulatory factors in the biosynthesis of red hair pigments: a new benzothiazole intermediate in the iron or copper assisted oxidation of 5-S-cysteinyldopa. Biochim. Biophys. Acta *1571*, 157-166

Edge, R., d'Ischia, M., Land, E. J., Napolitano, A., Navaratnam, S., Panzella, L., Pezzella, A., Ramsden, C.A., and Riley P.A. (2006). Dopaquinone redox exchange with dihydroxyindole and dihydroxyindole carboxylic acid. Pigment Cell Res. *19*, 443-450.

Erlenmeyer, von H., Junod, J. Guex, W., and Erne, M. (1948). Über carbonsäuren des thiazols. Helv. Chim. Acta *31*, 1342-1349

Fattorusso, E., Minale, L., De Stefano, S., Cimino, G., and Nicolaus, R.A. (1968). Struttura e biogenesi delle feomelanine. V. Sulla struttura della gallofeomelanina-1. Gazz. Chim. Ital. *98*, 1443-1463.

Greco G., Panzella, L., Napolitano, A., and d' Ischia, M. (2009a). Biologically inspired one-pot access routes to 4-hydroxybenzothiazole amino acids, red hair-specific markers of UV susceptibility and skin cancer risk. Tetrahedron Lett. *50*, 3095-3097.

Greco, G., Wakamatsu, K., Panzella, L., Ito, S., Napolitano, A.,andd’Ischia, M. (2009b).

Isomeric cysteinyldopas provide a (photo)degradable bulk component and a robust structural element in red human hair pheomelanin. Pigment Cell Melanoma Res. *22*, 319–327.

# Ito, S., and Prota, G. (1977) A facile one-step synthesis of cysteinyldopas using mushroom tyrosinase Experientia *33*, 1118-1119.

[Ito, S., Fujita, K., Yoshioka, M., Sienko, D., Nagatsu, T. (1986) Identification of 5-S- and 2-S-cysteinyldopamine and 5-S-glutathionyldopamine formed from dopamine by high-performance liquid chromatography with electrochemical detection.](https://scifinder.cas.org/scifinder/references/answers/A0C39B87X86F3507DX3B87EBF354241EBF6B:A0C73996X86F3507DX3B65D459536BCAA703/23.html?nav=eNpVkM8vA0EYhr9uIwiHchER4uAgJLNNVlsNCa360dhsRRFxkdFOatndWTPTai_CAQcXB-Xi0IOb3ok_QeIoXCTizlXiZHZLxJwm-Z555v3e-js0CQhgAQOJ8FRMi8ejq6PRGS0SjqVWtWQ0khqJxCNaNDmVSMTCmkQ3OIOOLVzCyMJOAaUdQQqEdb7Vrj4PjkcVCKShqYStIikzCP1xRtHeIOyoXu1tO3s9UQDKLgC0SOGmgJ7E8tJcZnE9baxMG0vyYmTWZxczywtpY1ZAq2m7lAlp4DuwB0H5DgQojP5PkqTUIth56Gf7j5dfHzLJ2m8S1-M5l_wwZQWUwxxRnsMMccJKhKE8tbHpoBy1beqgrPws65Lc-Ol1rffi9V4BRYd2u5JhedPB1jypCBjUpUiVItUXqQ2R2hCpDZEqyTEdmu2KZ-QCunUvrVoUpqXqprNN8nOYb2aJGCu7rgzX5S_jjdG_8bP1tFZ9GerzWvtd2ad-5nepw-r57c1I0Gt1t13WE5qYBP-UBQQ5x35xbfANNK-hCQ&key=caplus_1986:142685&title=SWRlbnRpZmljYXRpb24gb2YgNS1TLSBhbmQgMi1TLWN5c3RlaW55bGRvcGFtaW5lIGFuZCA1LVMtZ2x1dGF0aGlvbnlsZG9wYW1pbmUgZm9ybWVkIGZyb20gZG9wYW1pbmUgYnkgaGlnaC1wZXJmb3JtYW5jZSBsaXF1aWQgY2hyb21hdG9ncmFwaHkgd2l0aCBlbGVjdHJvY2hlbWljYWwgZGV0ZWN0aW9u&launchSrc=reflist&p=1) J. Chromatogr., Biomedical Applications *375,* 134-40

Liu, Y., Hong, L., Wakamatsu, K., Ito, S., Adhyaru, B., Cheng, C.Y., Bowers, C.R., and Simon, J.D. (2005a). Comparison of structural and chemical properties of black and red human hair melanosomes. Photochem. Photobiol. *81*, 135–144.

[Liu, Y](http://www.ncbi.nlm.nih.gov/pubmed?term=Liu%20Y%5BAuthor%5D&cauthor=true&cauthor_uid=15701042)., [Hong, L](http://www.ncbi.nlm.nih.gov/pubmed?term=Hong%20L%5BAuthor%5D&cauthor=true&cauthor_uid=15701042)., [Wakamatsu, K](http://www.ncbi.nlm.nih.gov/pubmed?term=Wakamatsu%20K%5BAuthor%5D&cauthor=true&cauthor_uid=15701042)., [Ito, S](http://www.ncbi.nlm.nih.gov/pubmed?term=Ito%20S%5BAuthor%5D&cauthor=true&cauthor_uid=15701042)., [Adhyaru, B.B](http://www.ncbi.nlm.nih.gov/pubmed?term=Adhyaru%20BB%5BAuthor%5D&cauthor=true&cauthor_uid=15701042)., [Cheng, C.Y](http://www.ncbi.nlm.nih.gov/pubmed?term=Cheng%20CY%5BAuthor%5D&cauthor=true&cauthor_uid=15701042)., [Bowers, C.R](http://www.ncbi.nlm.nih.gov/pubmed?term=Bowers%20CR%5BAuthor%5D&cauthor=true&cauthor_uid=15701042), and [Simon, J.D](http://www.ncbi.nlm.nih.gov/pubmed?term=Simon%20JD%5BAuthor%5D&cauthor=true&cauthor_uid=15701042). (2005b). Comparisons of the structural and chemical properties of melanosomes isolated from retinal pigment epithelium, iris and choroid of newborn and mature bovine eyes. [Photochem. Photobiol.](http://www.ncbi.nlm.nih.gov/pubmed/15701042" \o "Photochemistry and photobiology.) *81*, 510-516.

Menon, I. A., Haberman, H.F. (1974) Isolation of melanin granules. Methods Enzymol. *31*, 389-94.

Meredith, P., Powell, B.J., Riesz, J., Nighswander-Rempel, S.P., Pederson, M.R. and Moore, E.G. (2006) *Soft Matter* *2* 37-44.

Mostert, A.B., Powell, B.J., Gentle, I.R. and Meredith, P. (2012) Appl. Phys. Letts *100,* 093701

Napolitano, A., De Lucia, M., Panzella, L., and d’Ischia, M. (2008). The “Benzothiazine” chromophore of pheomelanins: a reassessment Photochem. Photobiol., *84*, 593-599.

Napolitano, A., Pezzella, A., Vincensi M. R., and Prota G.(1995). Oxidative degradation of melanins to pyrrole acids: a model study. Tetrahedron *51*, 5913-5920.

Napolitano, A., Vincensi, M. R., d’Ischia, M., and Prota, G. (1996). A new benzothiazole derivative by degradation of pheomelanins with alkaline hydrogen peroxideTetrahedron Lett. *37*, 6799-6802

Novellino, L., Napolitano, A., and Prota, G. (2000). Isolation and characterization of mammalian eumelanins from hair and irides. Biochim. Biophys. Acta *1475*, 295–306.

Palumbo , A., Napolitano, A., De Martino, L., Vieira W., Hearing, V.J. (1994) Specific incorporation of 2-thiouracil into biological melanins. Biochim. Biophys Acta 1200, 271-276.

Panzella, L., Manini, P., Monfrecola, G., d’Ischia, M., and Napolitano, A. (2007). An easy-to-run method for routine analysis of eumelanin and pheomelanin in pigmented tissues. Pigment Cell Res*.*

*20*, 128–133.

Panzella, L., Szewczyk, G., d'Ischia, M., Napolitano, A**.**, and Sarna, T. (2010). Zinc-induced structural effects enhance oxygen consumption and superoxide generation in synthetic pheomelanins on UVA/Visible light irradiationPhotochem. Photobiol***.***  *86*, 757-764.

Patil, D. P., and Chedekel, M. R. (1984).Synthesis and analysis of pheomelanin degradation products. 2 J. Org. Chem. *49*, 997-1000.

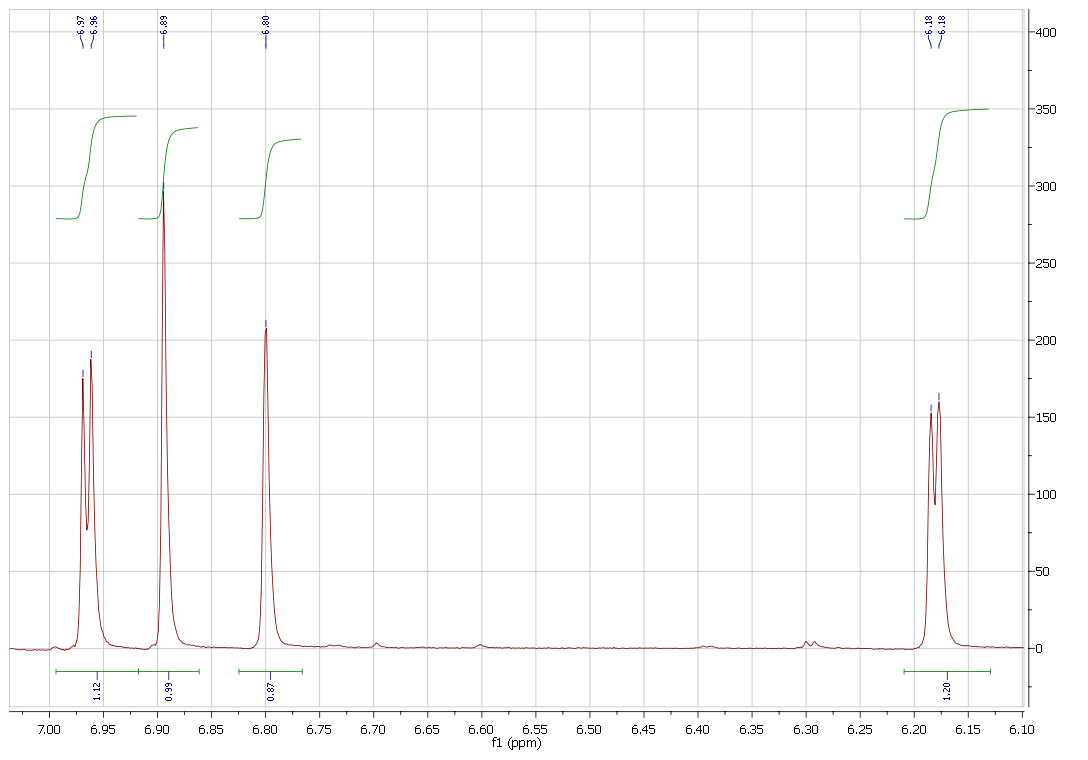
[Sarna, T](http://www.ncbi.nlm.nih.gov/pubmed?term=Sarna%20T%5BAuthor%5D&cauthor=true&cauthor_uid=12589778)., [Burke, J.M](http://www.ncbi.nlm.nih.gov/pubmed?term=Burke%20JM%5BAuthor%5D&cauthor=true&cauthor_uid=12589778)., [Korytowski, W](http://www.ncbi.nlm.nih.gov/pubmed?term=Korytowski%20W%5BAuthor%5D&cauthor=true&cauthor_uid=12589778)., [Rózanowska, M](http://www.ncbi.nlm.nih.gov/pubmed?term=R%C3%B3zanowska%20M%5BAuthor%5D&cauthor=true&cauthor_uid=12589778)., [Skumatz, C.M](http://www.ncbi.nlm.nih.gov/pubmed?term=Skumatz%20CM%5BAuthor%5D&cauthor=true&cauthor_uid=12589778)., [Zareba, A](http://www.ncbi.nlm.nih.gov/pubmed?term=Zareba%20A%5BAuthor%5D&cauthor=true&cauthor_uid=12589778)., and [Zareba, M](http://www.ncbi.nlm.nih.gov/pubmed?term=Zareba%20M%5BAuthor%5D&cauthor=true&cauthor_uid=12589778). (2003). Loss of melanin from human RPE with aging: possible role of melanin photooxidation. [Exp. Eye Res.](http://www.ncbi.nlm.nih.gov/pubmed/12589778" \o "Experimental eye research.) *76*, 89-98.

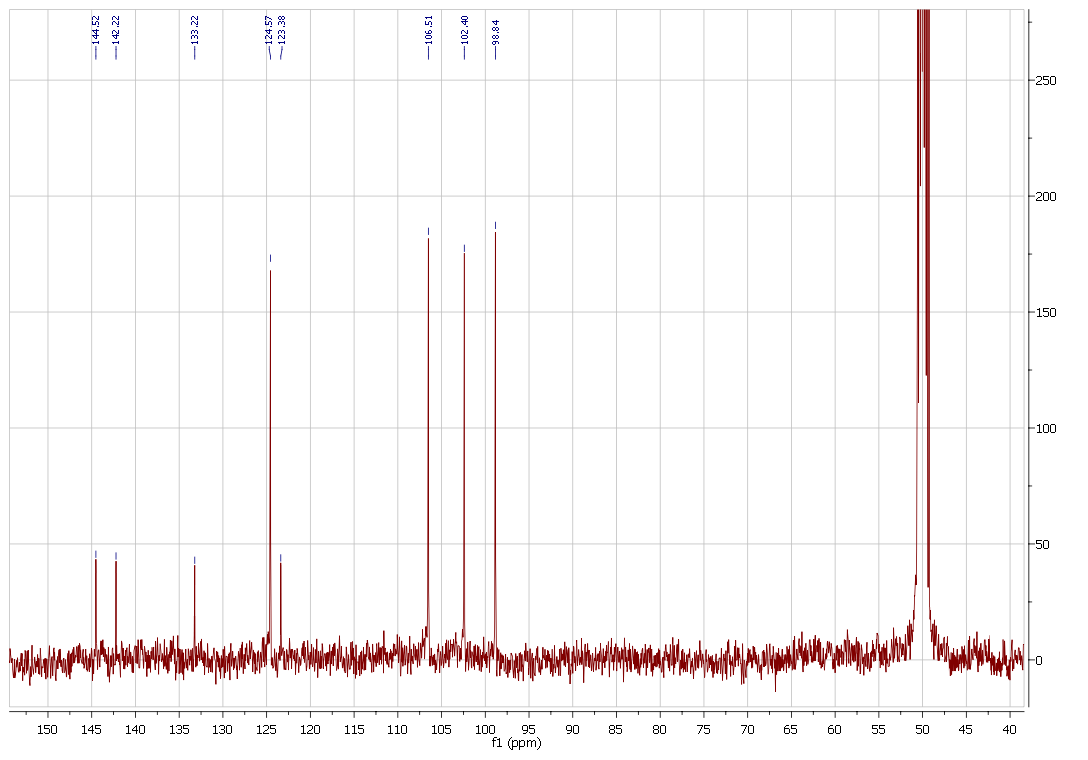
[Wakamatsu, K](http://www.ncbi.nlm.nih.gov/pubmed?term=Wakamatsu%20K%5BAuthor%5D&cauthor=true&cauthor_uid=12887698), [Fujikawa, K](http://www.ncbi.nlm.nih.gov/pubmed?term=Fujikawa%20K%5BAuthor%5D&cauthor=true&cauthor_uid=12887698), [Zucca, F.A](http://www.ncbi.nlm.nih.gov/pubmed?term=Zucca%20FA%5BAuthor%5D&cauthor=true&cauthor_uid=12887698)., [Zecca, L](http://www.ncbi.nlm.nih.gov/pubmed?term=Zecca%20L%5BAuthor%5D&cauthor=true&cauthor_uid=12887698)., and [Ito, S](http://www.ncbi.nlm.nih.gov/pubmed?term=Ito%20S%5BAuthor%5D&cauthor=true&cauthor_uid=12887698). (2003). The structure of neuromelanin as studied by chemical degradative methods**.** [J Neurochem.](http://www.ncbi.nlm.nih.gov/pubmed/12887698" \o "Journal of neurochemistry.) *86,*1015-1023.

Wakamatsu K, Ito S. (1988) [Preparation of eumelanin-related metabolites 5,6-dihydroxyindole, 5,6-dihydroxyindole-2-carboxylic acid, and their O-methyl derivatives.](http://www.ncbi.nlm.nih.gov/pubmed/3394933) Anal Biochem. *170*, 335-340

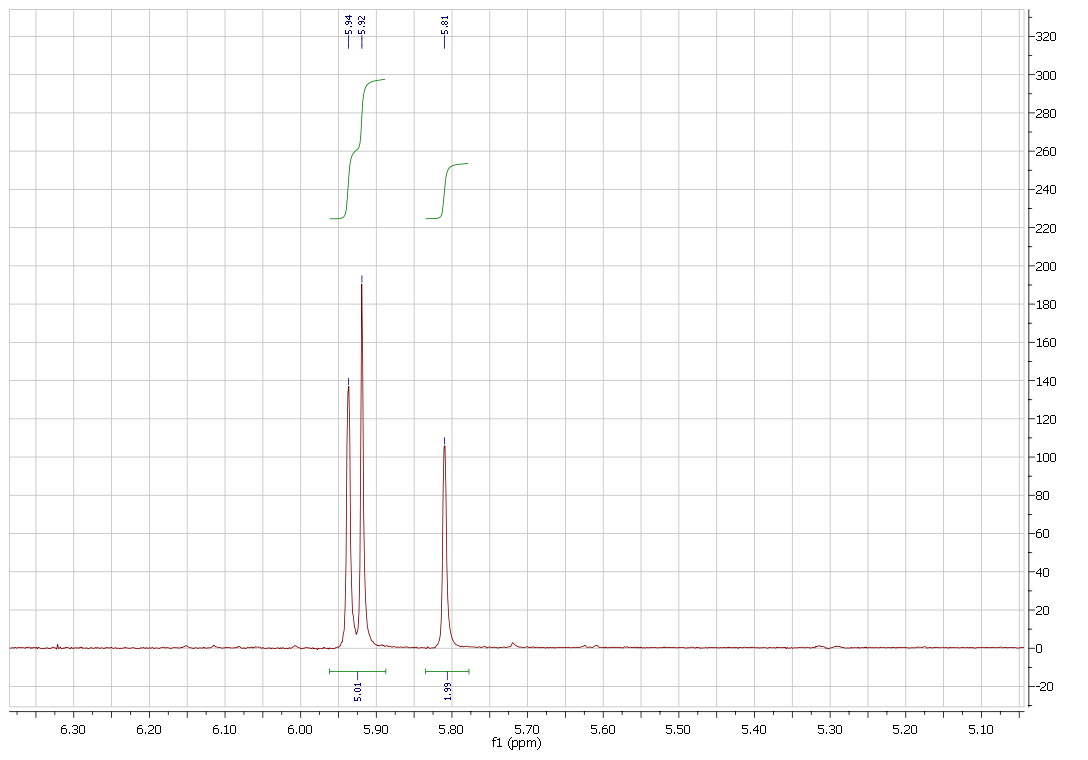
Wakamatsu K, Ohtara K, and Ito S. (2009). [Chemical analysis of late stages of pheomelanogenesis: conversion of dihydrobenzothiazine to a benzothiazole structure.](http://www.ncbi.nlm.nih.gov/pubmed/19493317) Pigment Cell Melanoma Res. *22*, 474-486

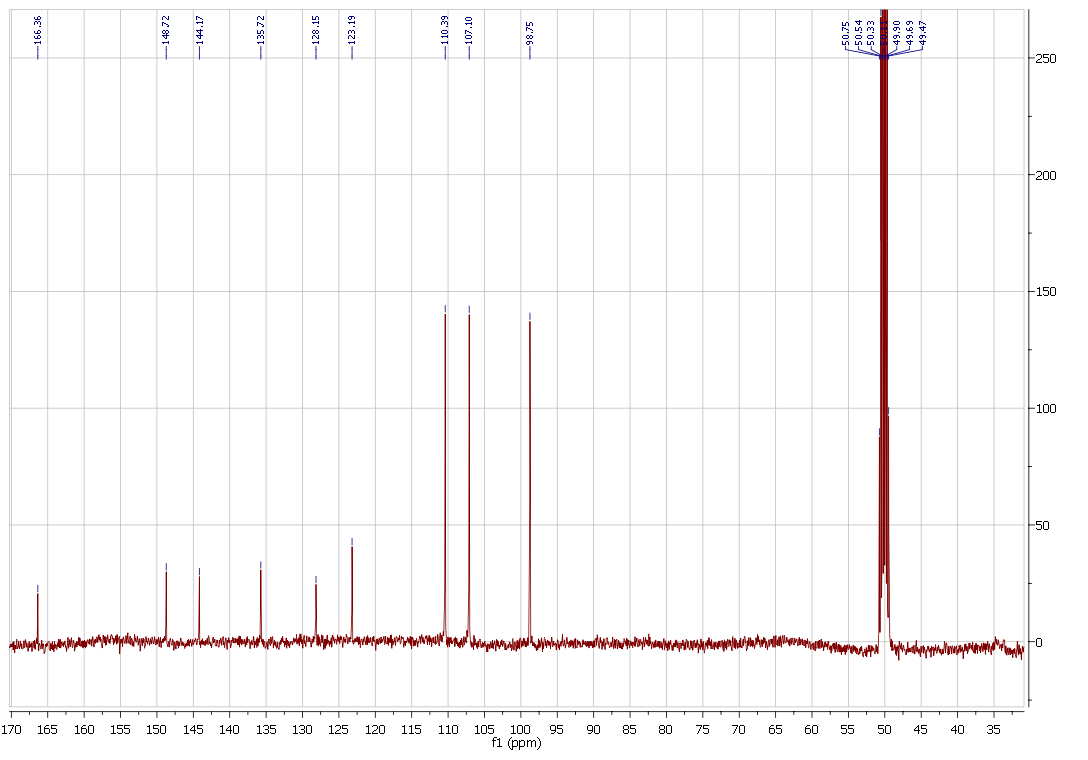
Watt, A. A.R, Bothma, J. and Meredith, P. (2009) Soft Matter *5,*  3754-3760.





*Fig S1 1H (top) and 13C (bottom) NMR spectrum of DHI in methanol–d4*

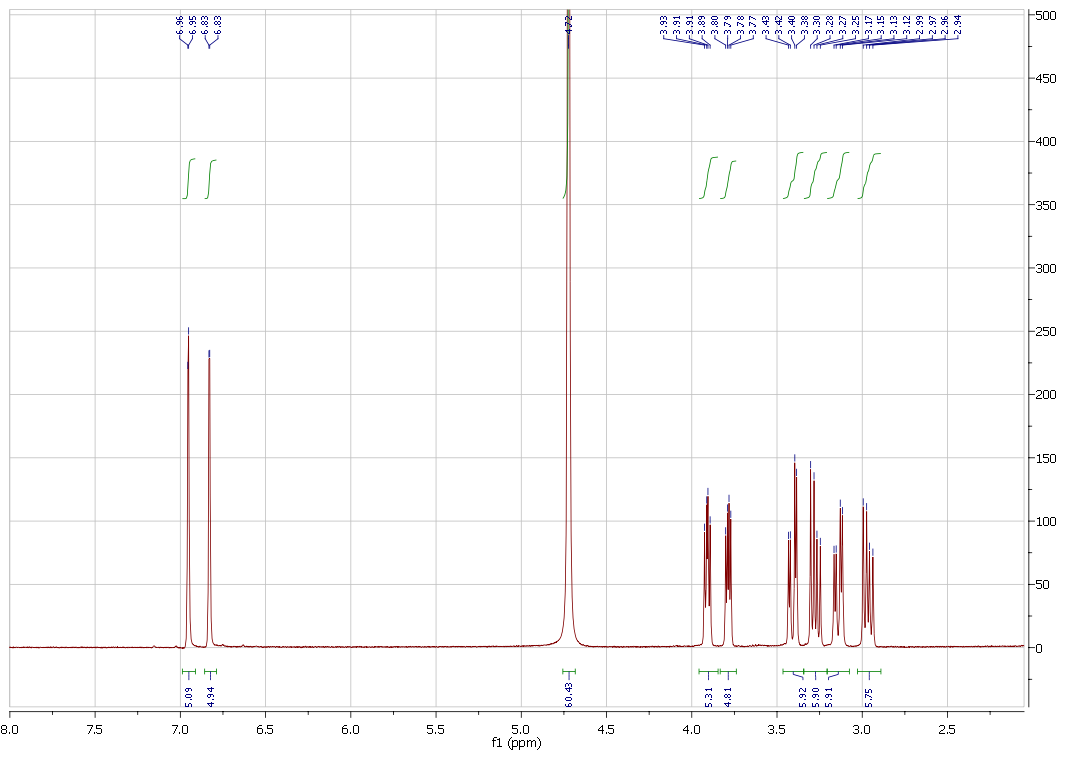
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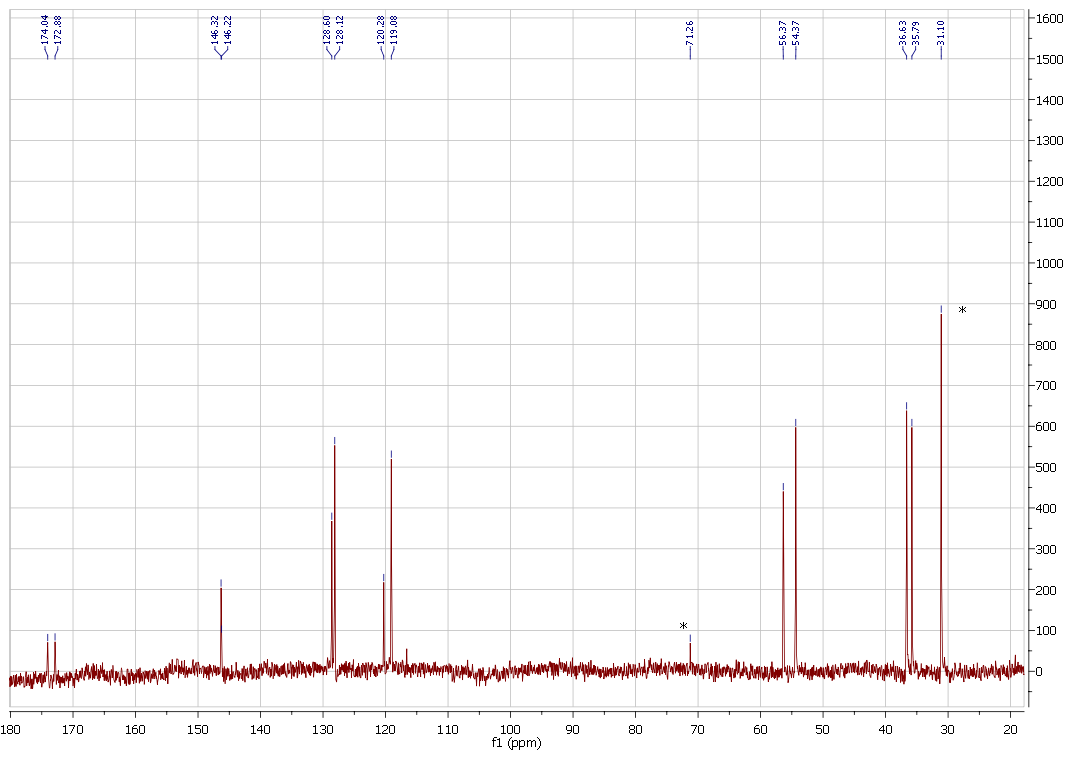


*Fig S2 1H (top) and 13C (bottom) NMR spectrum of DHICA in methanol–d4*

|  |  |
| --- | --- |
| Residual DOPA  5SCD  *280 nm*  Isomeric CDs | 5SCD  *254 nm*  5SCD |

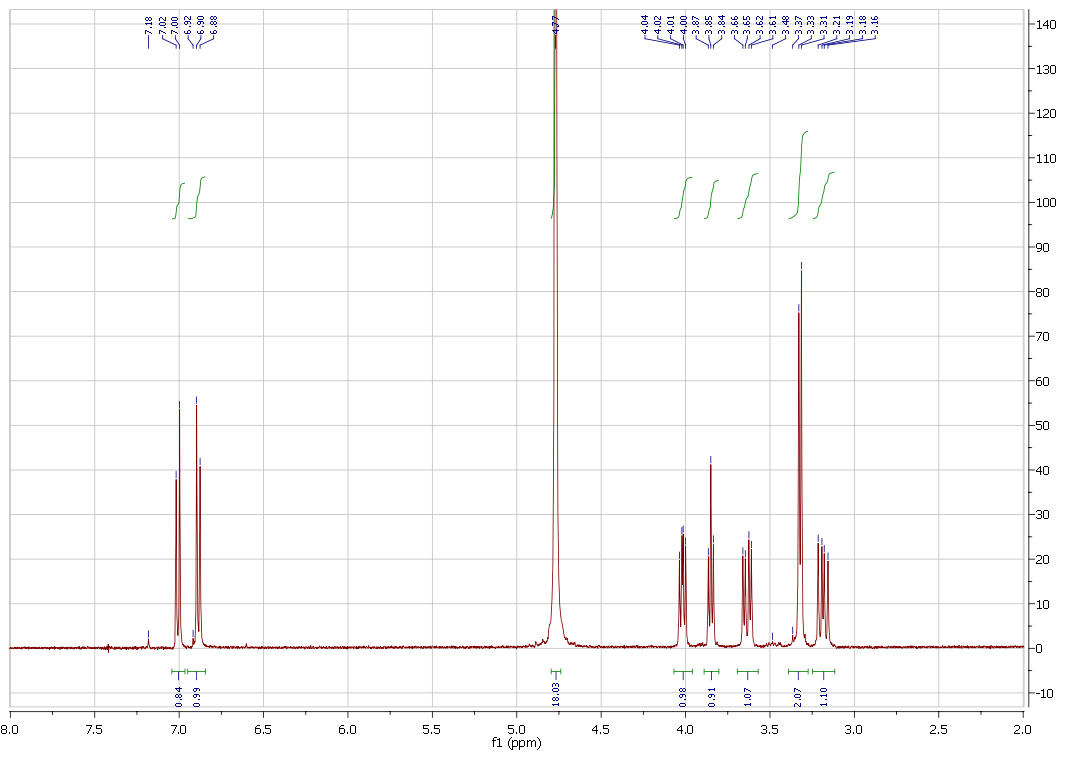
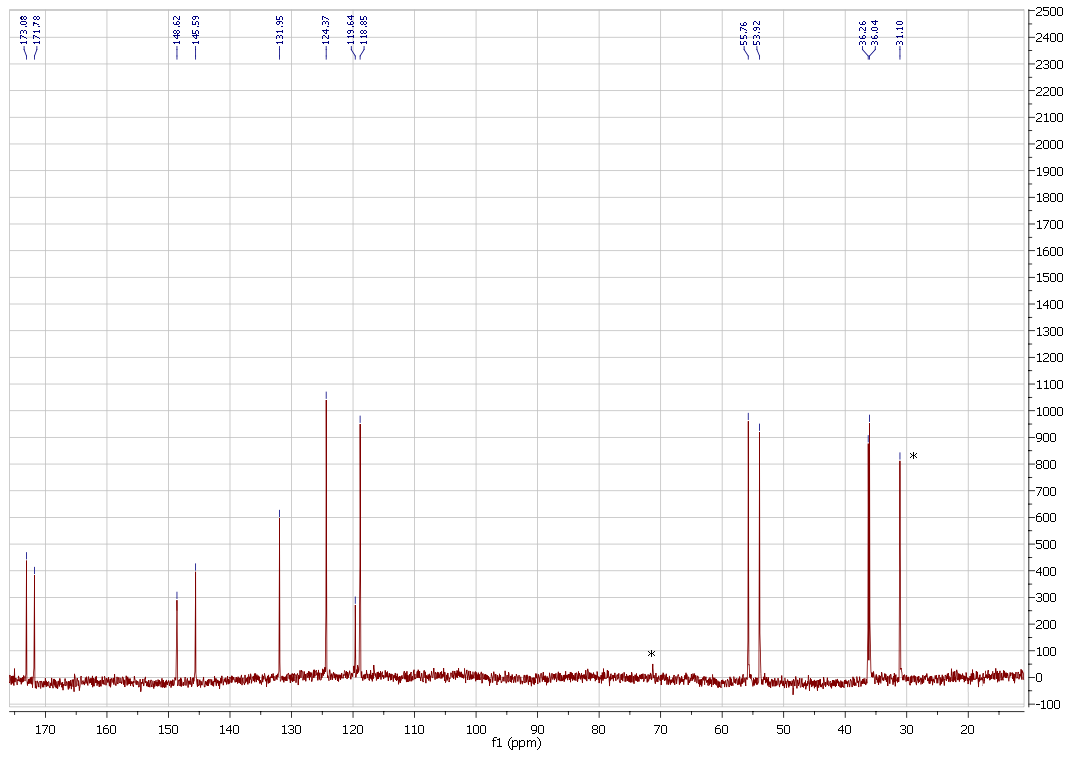
*Fig S3 HPLC analysis of the reaction mixture from the synthesis of 5SCD at two different wavelengths before (top panels) and after (bottom panels) the first ion exchange chromatography purification*

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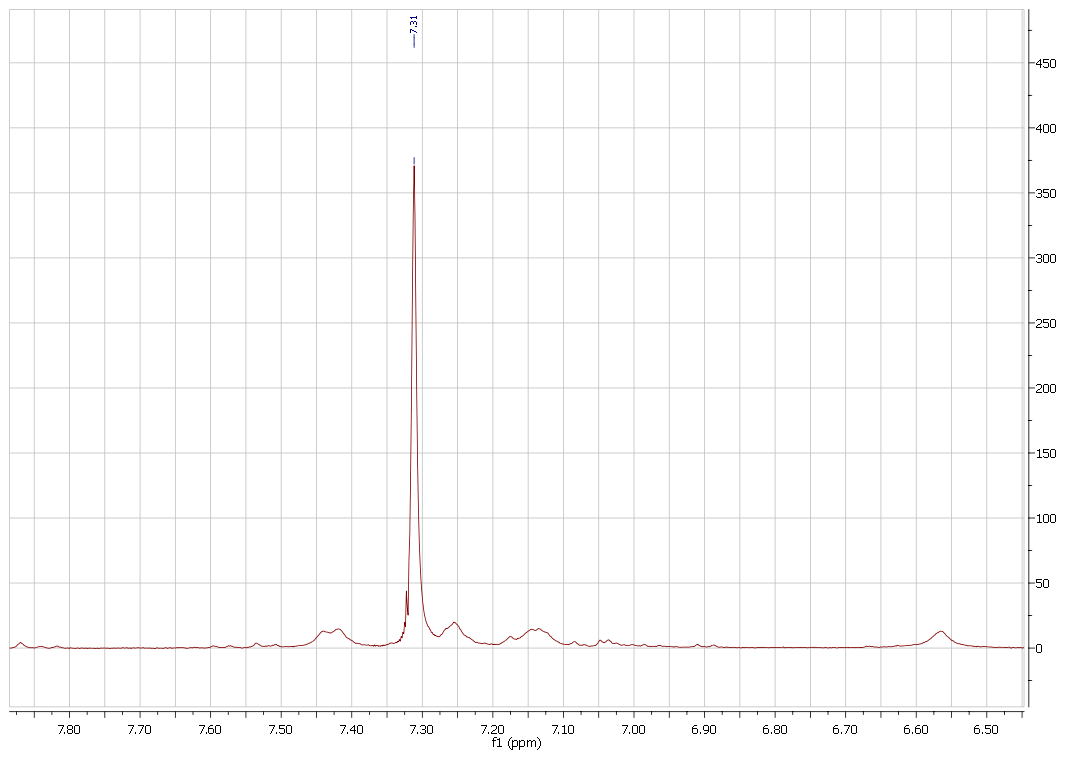


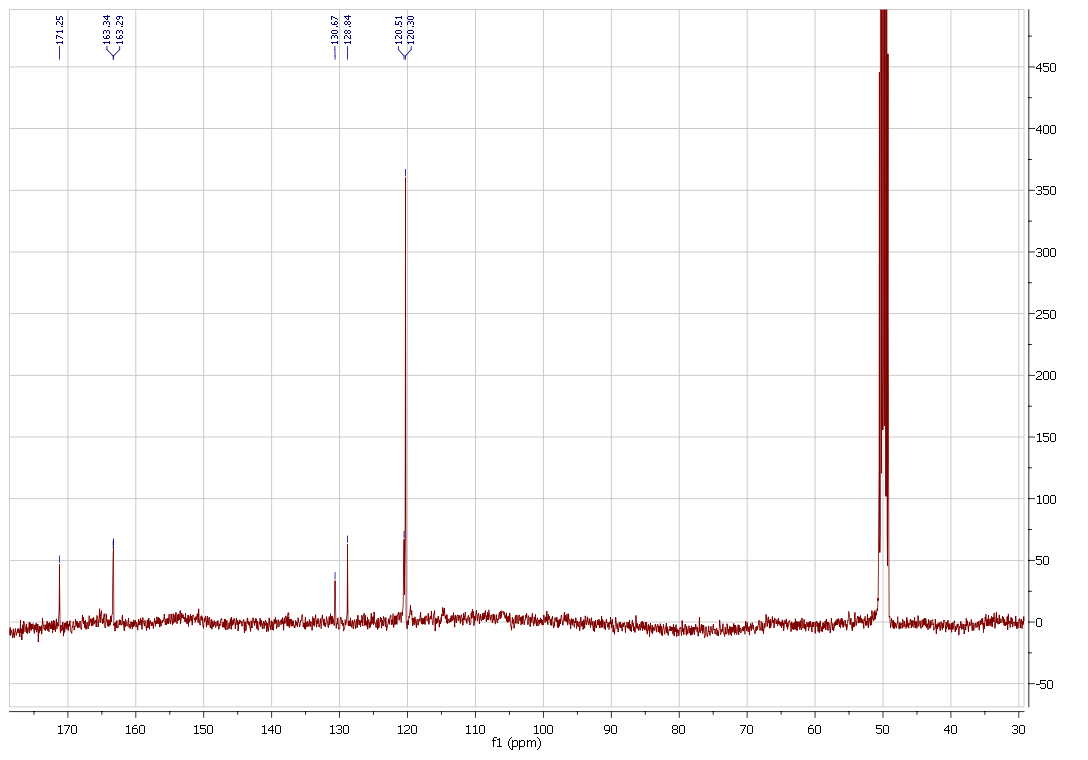
*Fig S4. 1H (top) and 13C NMR (bottom) spectrum of 5SCD in D2O. Peaks marked are due to t-butanol used as an internal reference*

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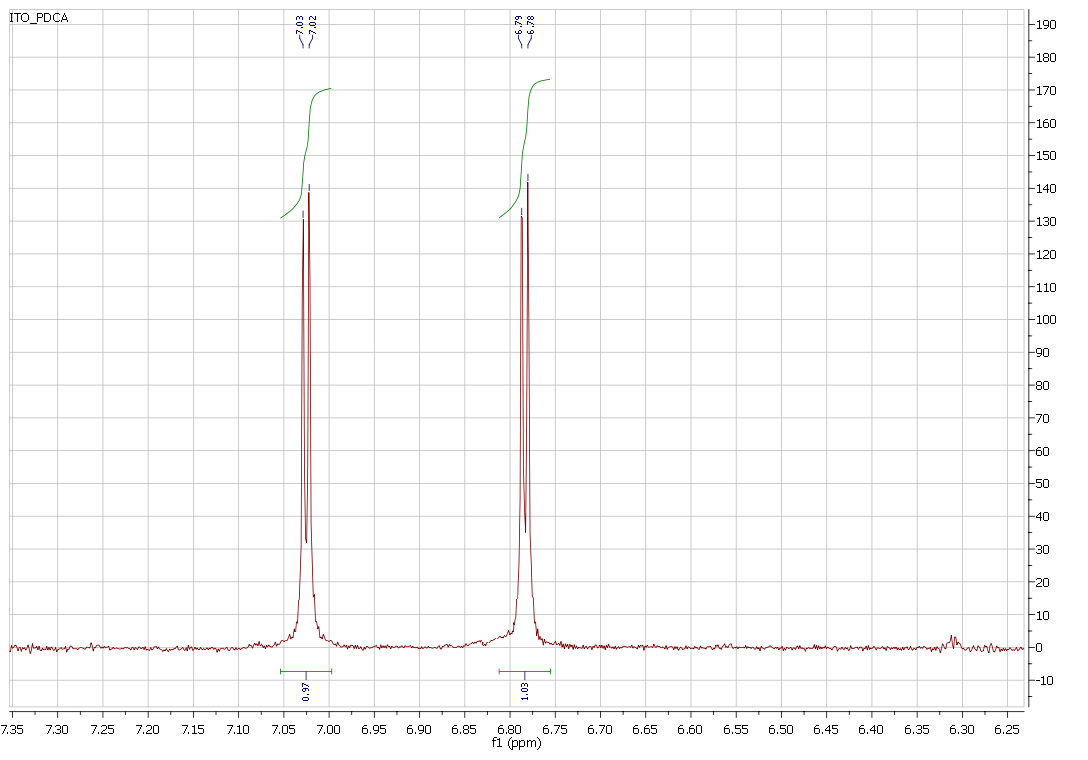


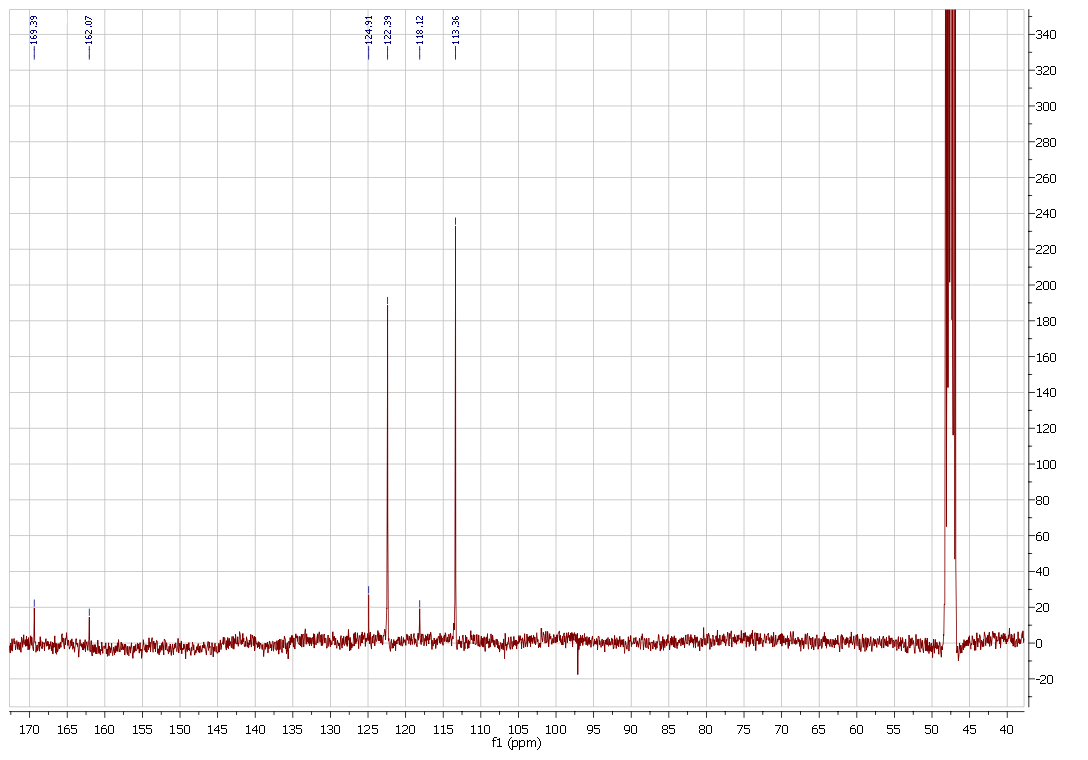
*Fig S5. 1H (top) and 13C NMR (bottom) spectrum of 2SCD in D2O. Peaks marked are due to t-butanol used as an internal reference*



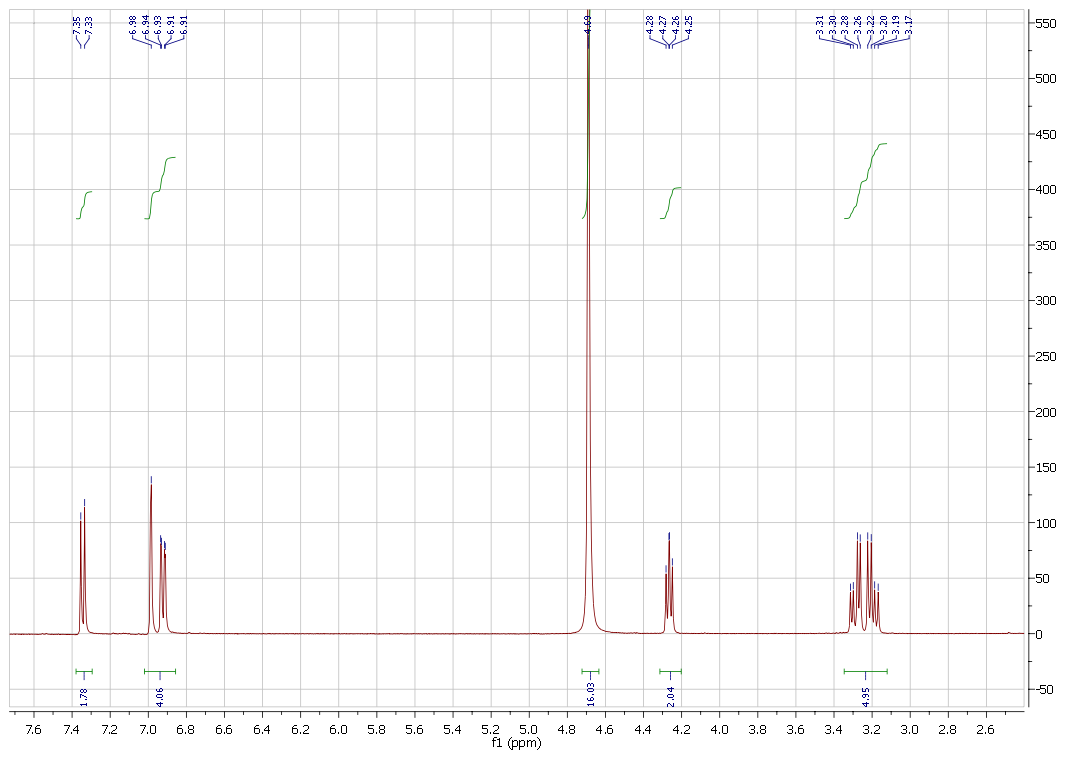


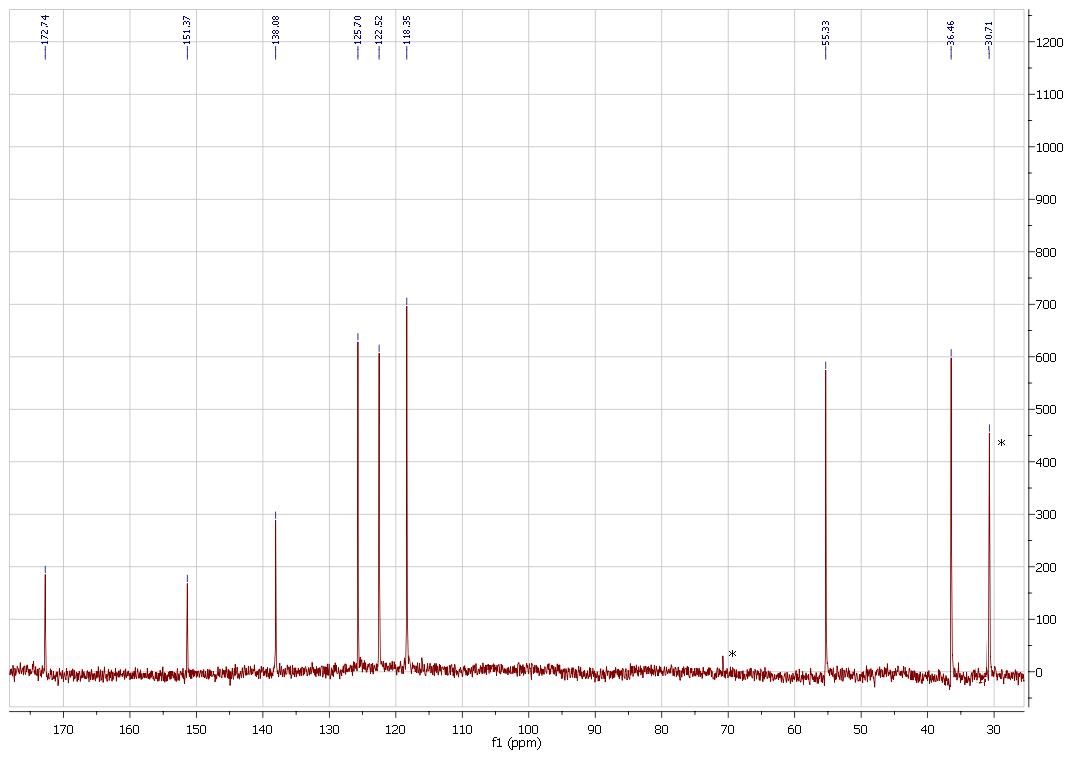
*Fig S6. 1H (top) and 13C NMR (bottom) spectrum of PTCA in methanol-d4.*



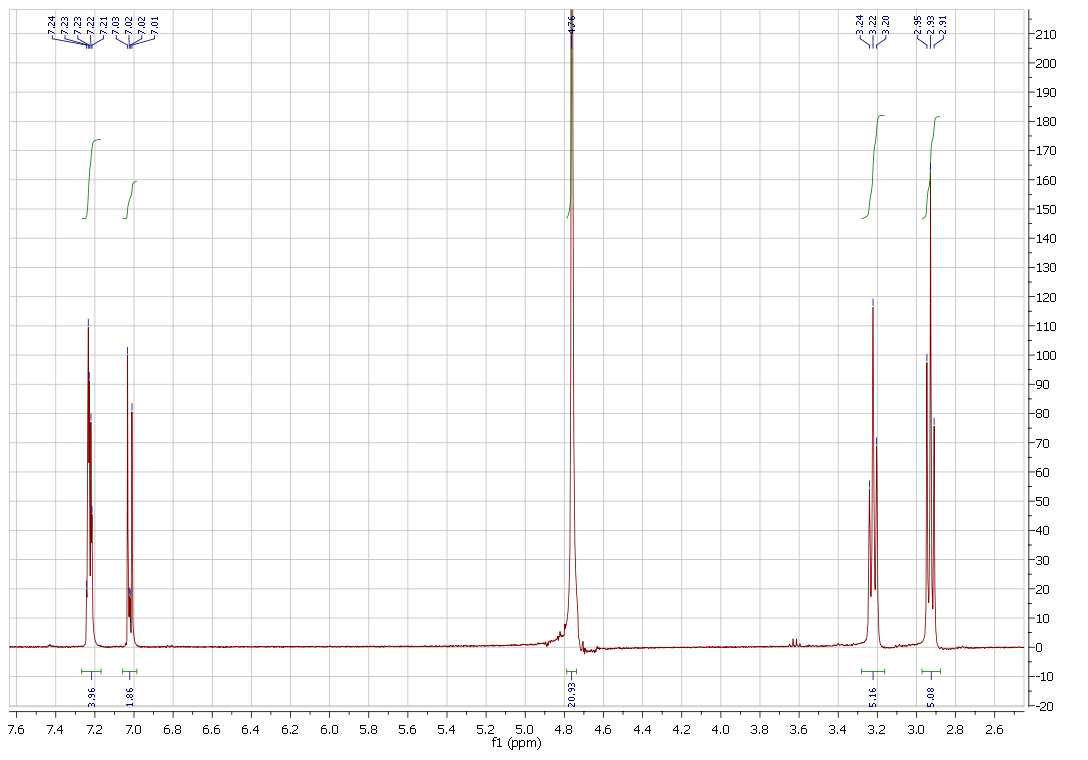


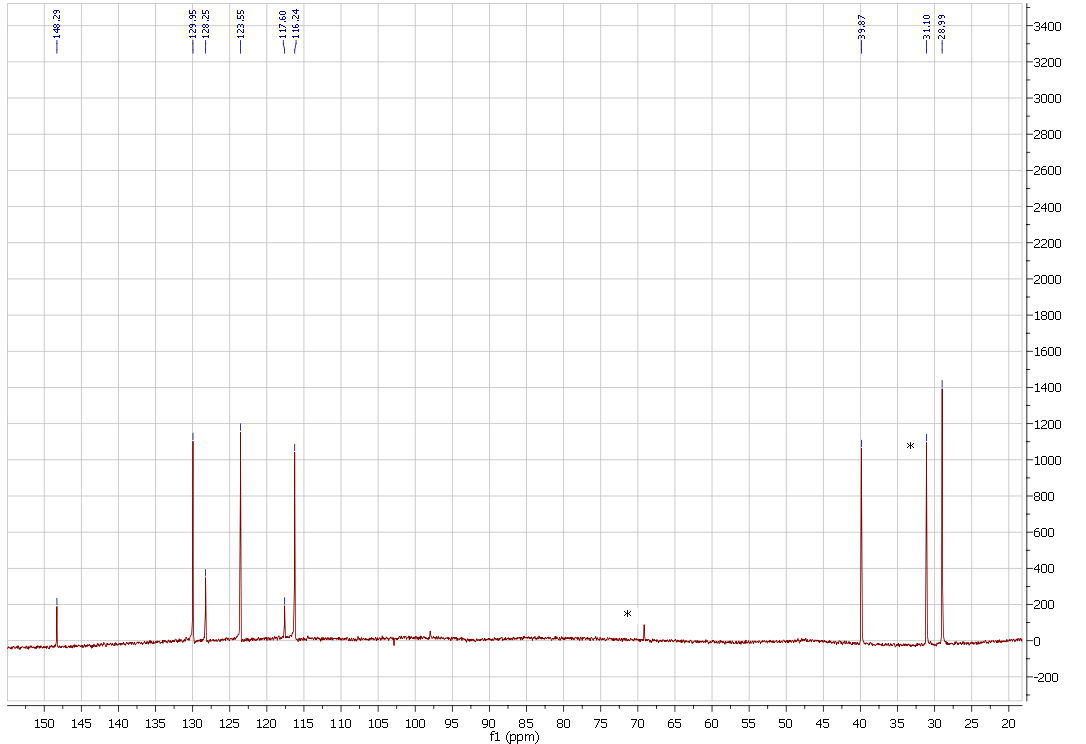
*Fig S7. 1H (top) and 13C NMR (bottom) spectrum of PDCA in methanol-d4.*



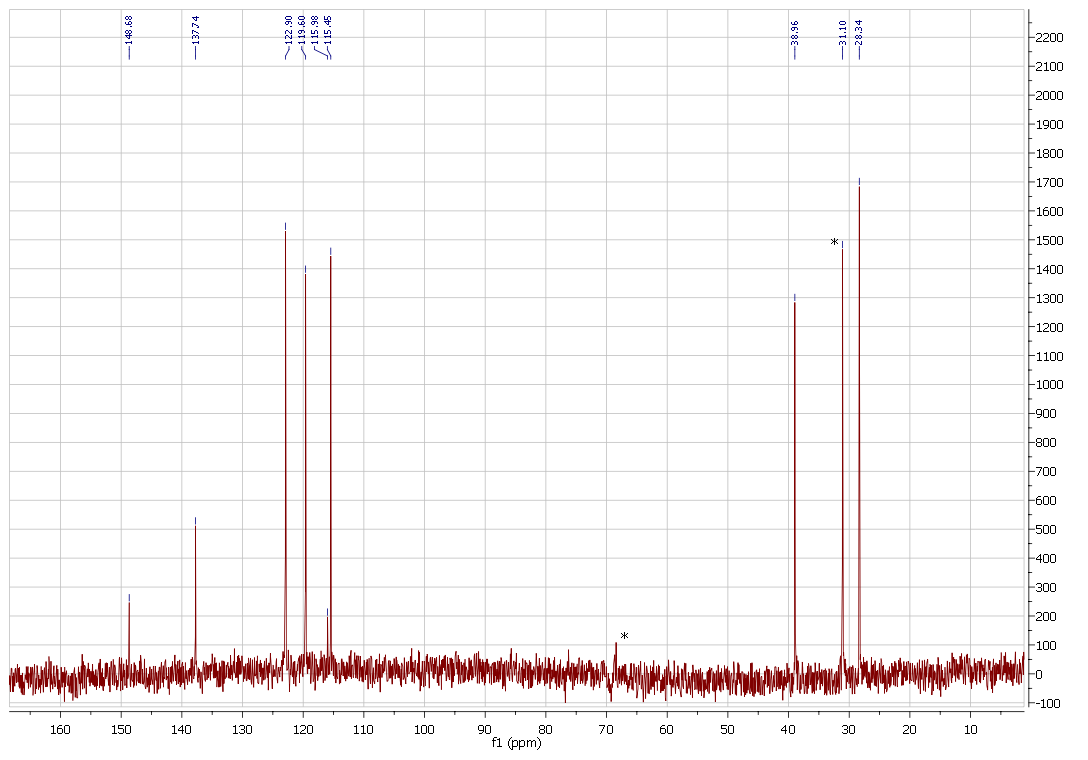
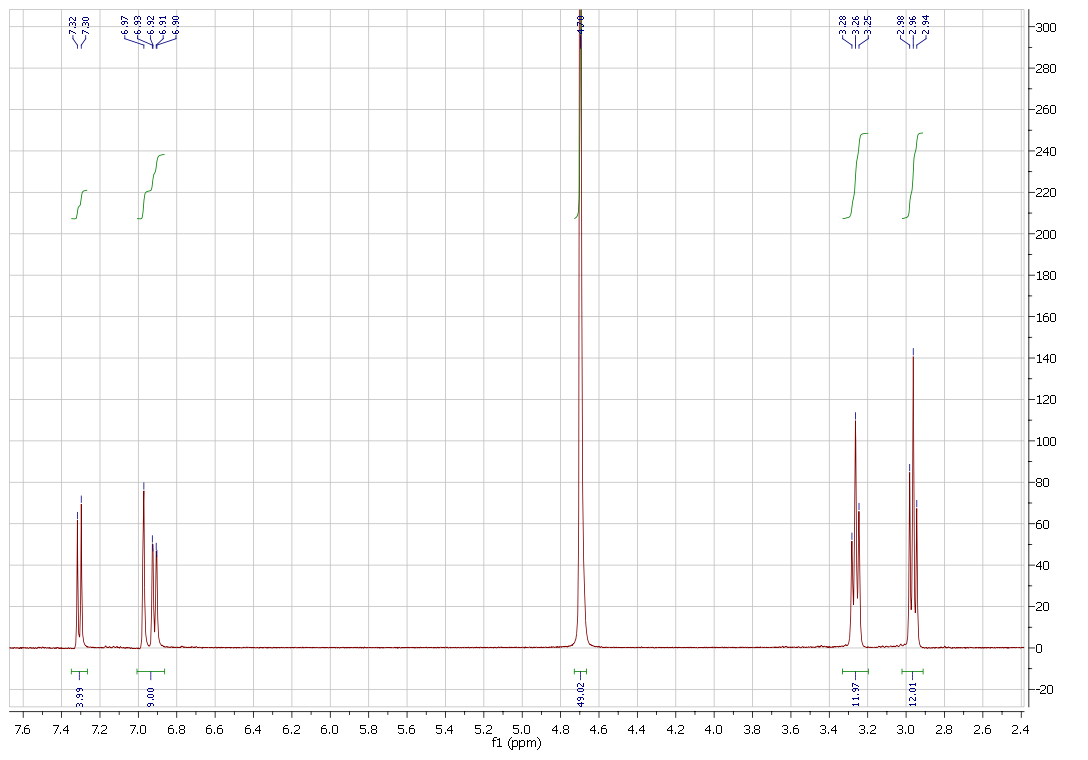


*Fig S8. 1H (top) and 13C (bottom) NMR spectrum of 4AHP in D2O. Marked peaks are due to t-butanol added as internal reference.*

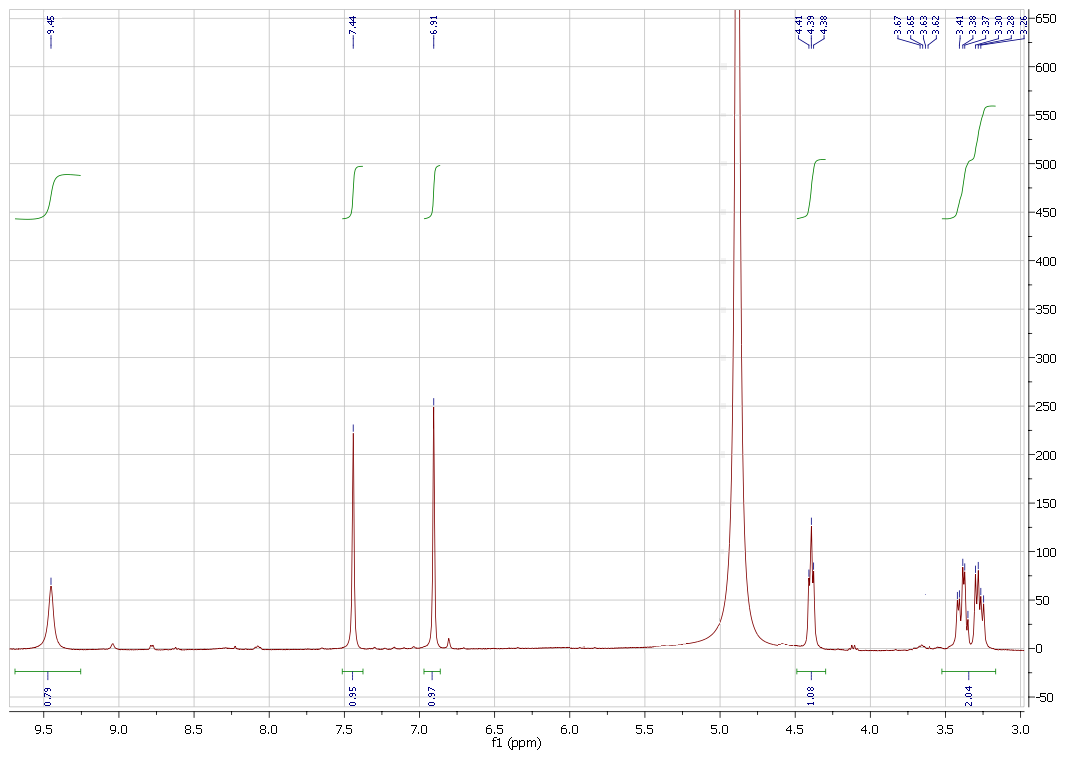


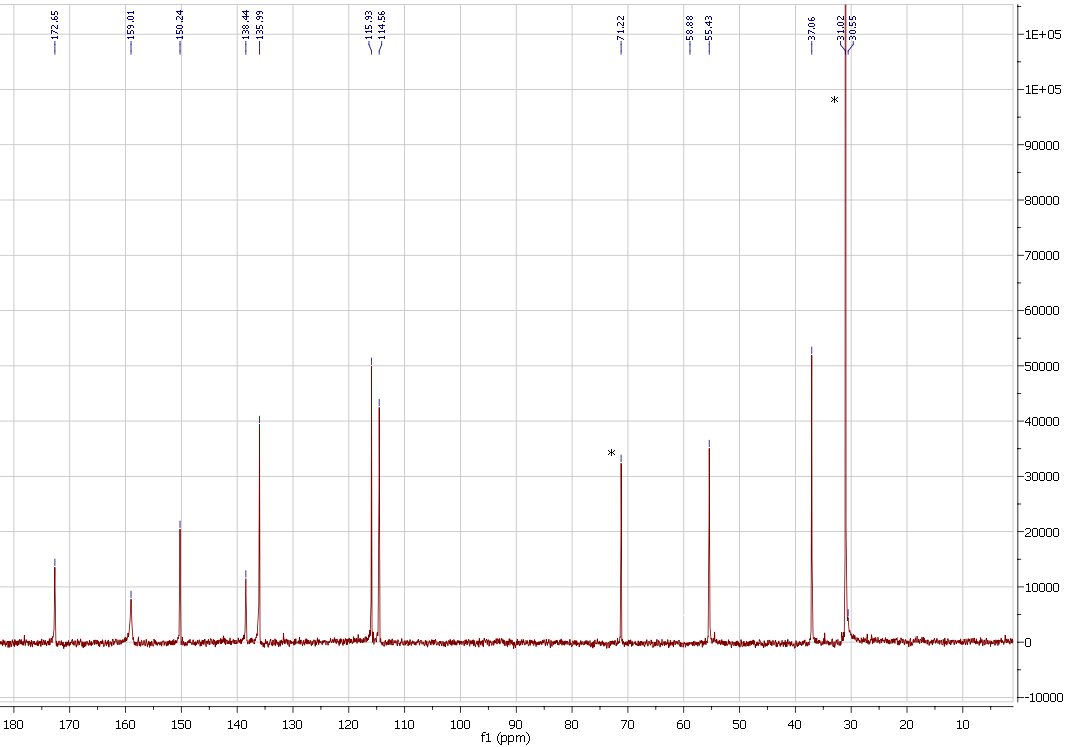
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*Fig S9. 1H (top) and 13C NMR (bottom) spectrum of 3AHPEA in D2O. Marked peaks are due to t-butanol added as internal reference.*

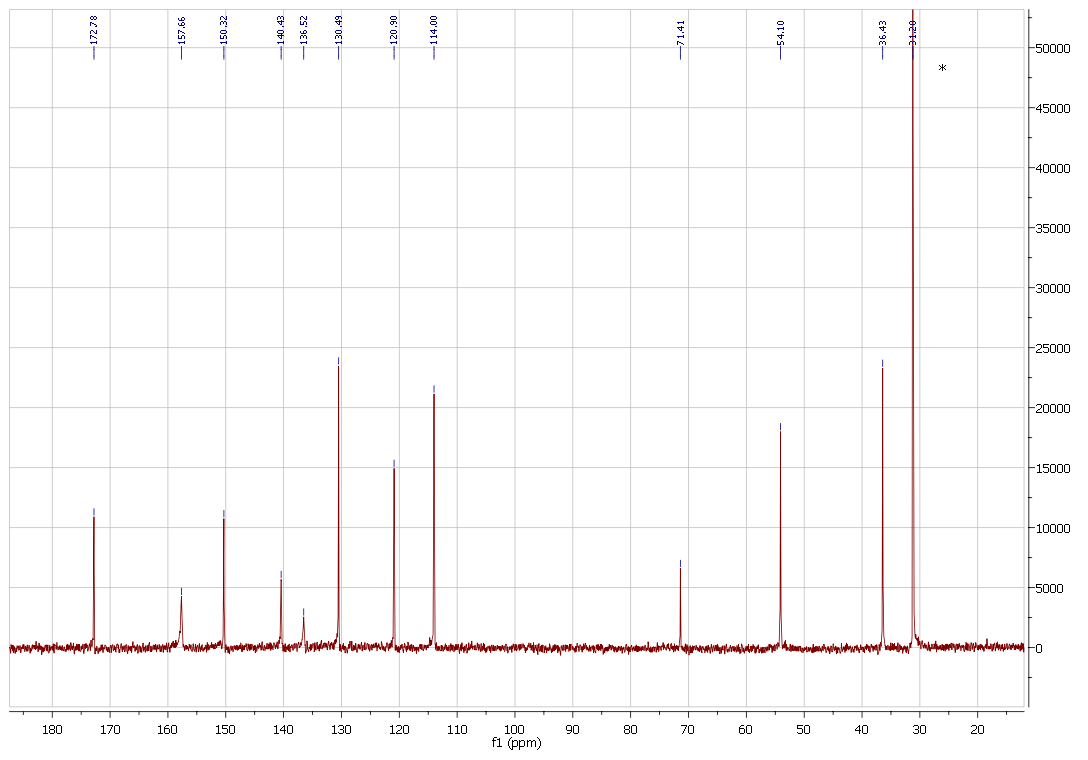
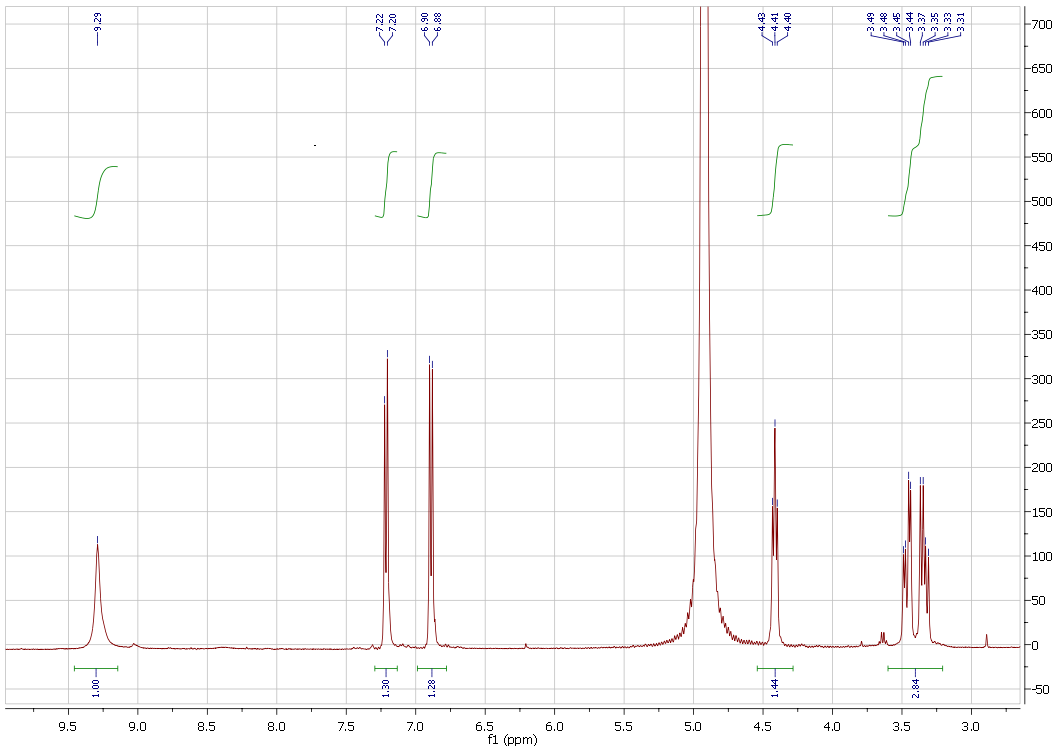


*Fig S10. 1H (top) and 13C NMR (bottom) spectrum of 4AHPEA in D2O. Marked peaks are due to t-butanol used as internal reference.*

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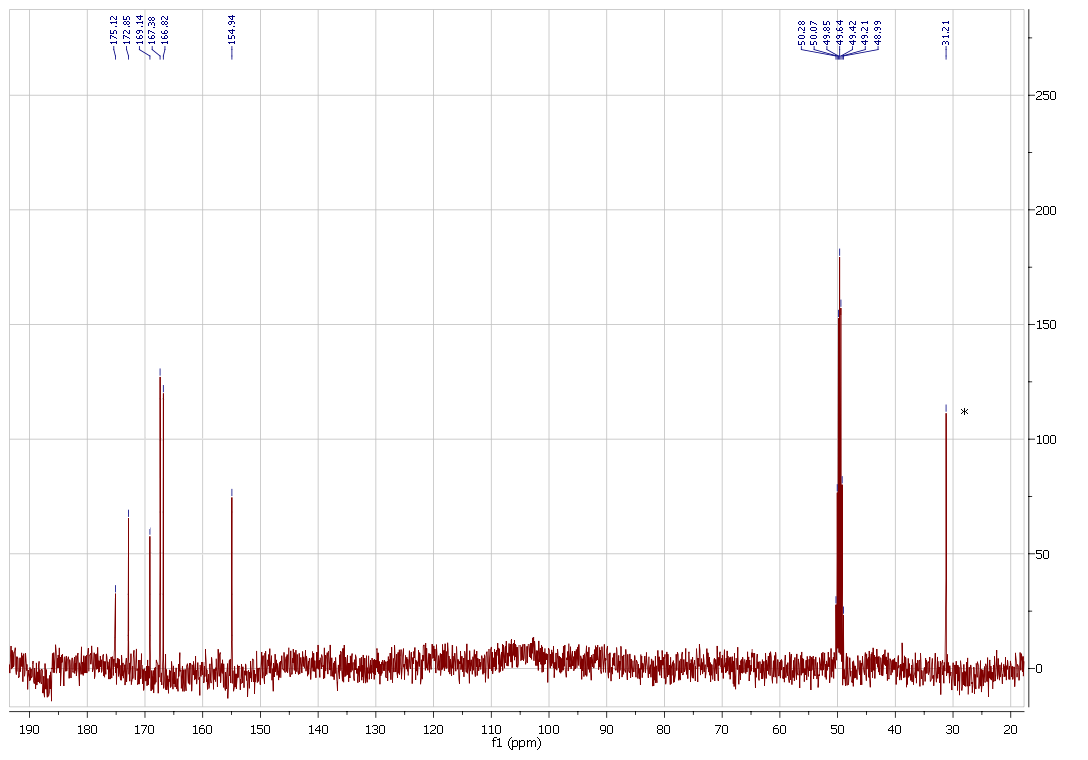
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*Fig S11 1H (top) and 13C NMR (bottom) spectrum of BT in D2O. Marked peaks are due to t-butanol used as internal reference*

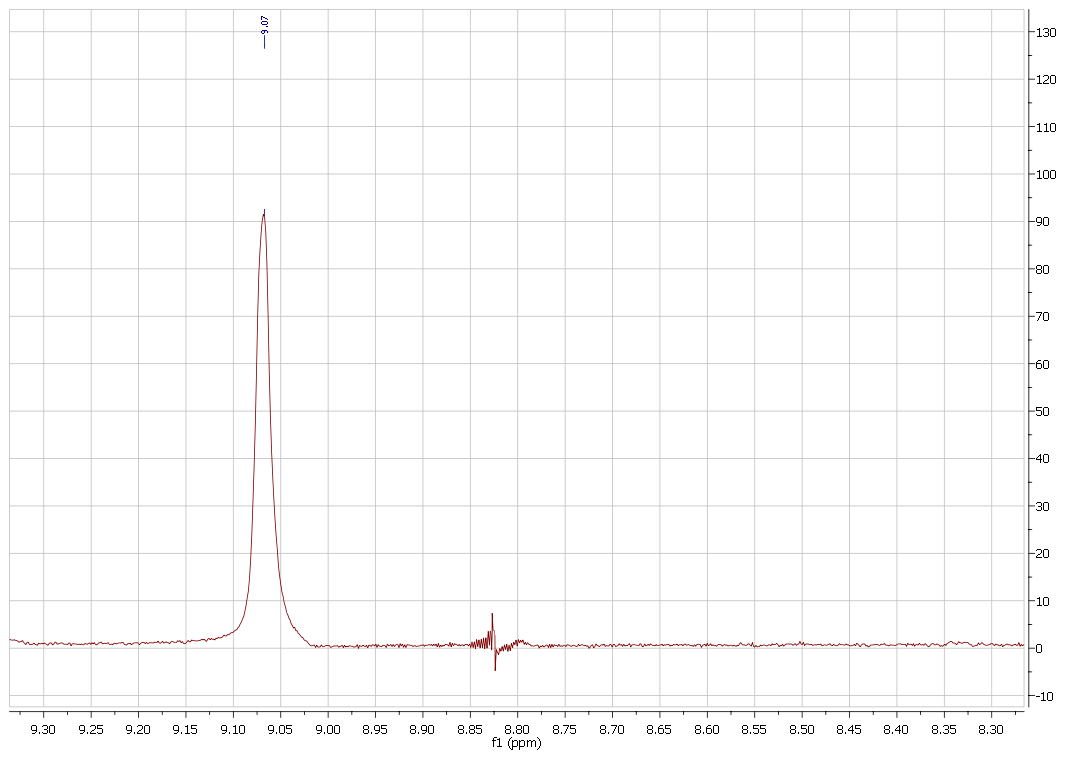
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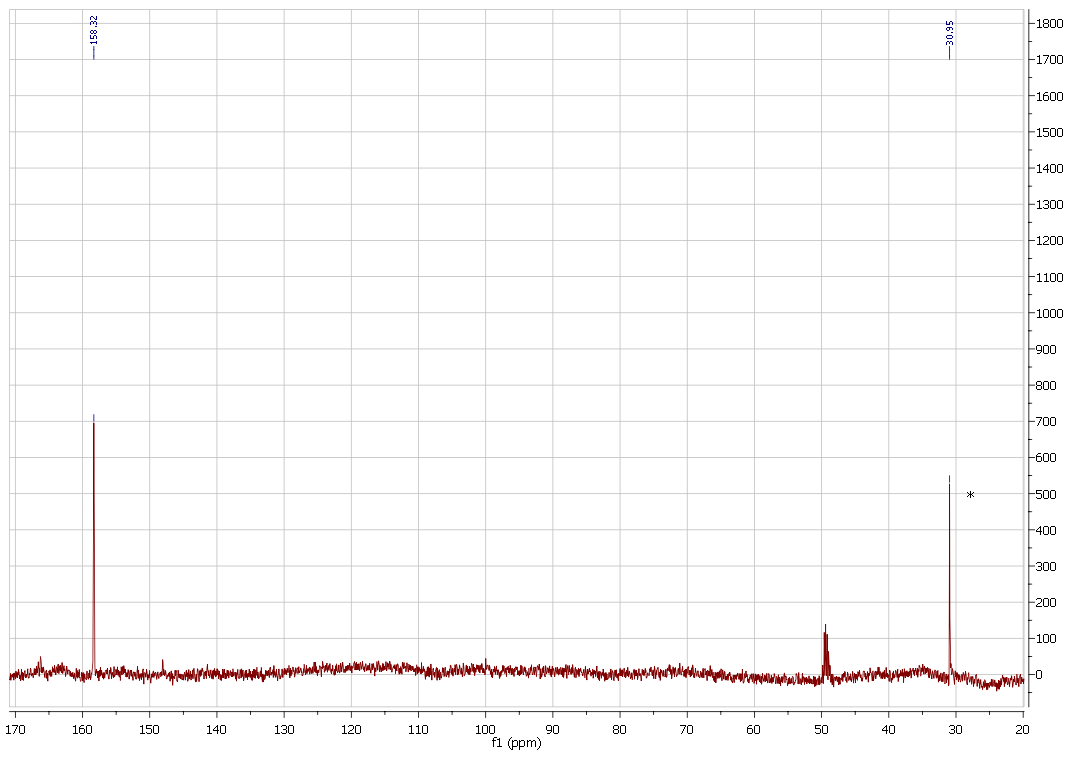
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*Fig S12 1H (top) and 13C NMR (bottom) spectrum of BT2 in D2O. Marked peaks are due to t-butanol used as internal reference*

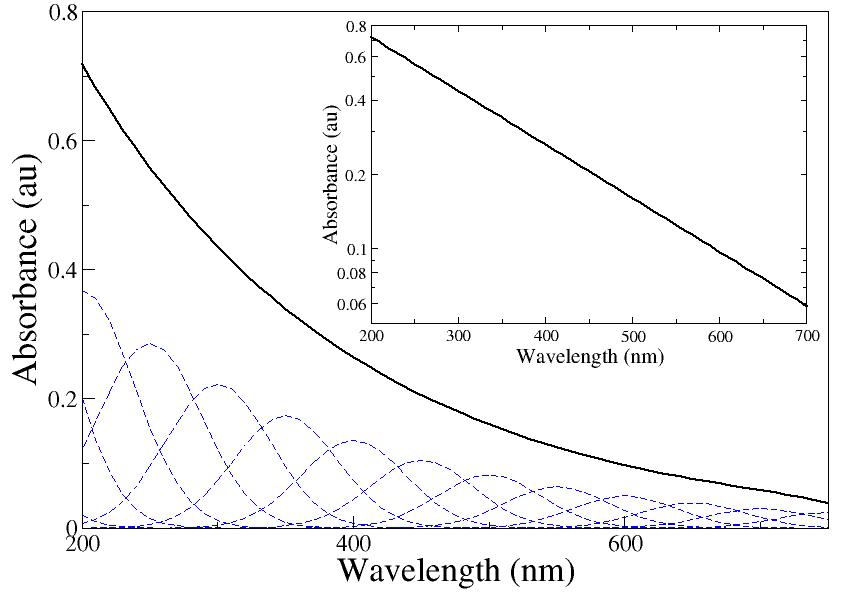


*Fig S13. 13C NMR spectrum of TTCA.3K salt in methanol-d4. Marked peak is due to t-butanol used as internal reference*





*Fig S14. 1H (top) and 13C (bottom) NMR spectrum of TDCA in methanol-d4. Marked peak is due to to t-butanol used as internal reference*

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**Figure S15a**: The aqueous solution absorption spectrum of synthetic DOPA melanin at alkaline pH. The spectrum is broad, featureless and monotonic, and closely approximates an exponential dependence upon wavelength (inset). The smooth exponential profile is derived from an ensemble effect – the overlap of multiple chromophores with typical inhomogeneously broadened individual absorption peaks. Adapted from Meredith *et al.* Soft Matter 2006, 2, 37-44 (with permission).



**Figure S15b**: (Left) A transmission electron microscope (TEM) image of the structure of a melanin sample cast from disaggregated synthetic DOPA melanin. (Right) Absorption spectra of the thin film from the TEM micrograph (red) and a corresponding solution spectrum (black) of normal aggregated synthetic DOPA melanin. This indicates that the characteristic exponential optical absorption is due to the primary indole quinone structure. Adapted from Watt *et al*., Soft Matter 2009, 5, 3754 (with permission).



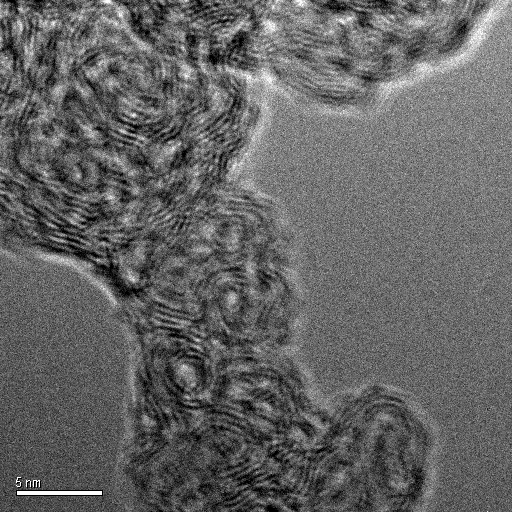
**Figure S16** The fluorescence spectra of synthetic DOPA melanin as a function of excitation wavelength progressively red (dotted and dashed). The shape of the emission spectra are characteristic of organic chromophores and the different peaks represent selection of a different species within the ensemble as a function of the excitation wavelength. Adapted from Meredith *et al*, Soft Matter 2006, 2, 37-44 (with permission).

Final fig1.tif

**Figure S17**: Electrical conductivity of a synthetic DOPA melanin pellet as a function of water content (hydration): (a) sandwich electrodes; and (b) a surface van der Pauw contact geometry. The measurement configurations are show as insets. Numerical fits to the modified dielectric theory (or Mott-Davis Amorphous Semiconductor (MDAS)) model are shown (solid blue lines). The qualitative agreement between the MDAS in the sandwich contact geometry is derived from the non-equilibrium water absorption behaviour, whereas the van der Pauw geometry which delivers the correct equilibrium behaviour shows no such agreement. Reprinted with permission from Mostert et al. Appl. Phys. Letts 100, 093701. Copyright 2012. American Institute of Physics.

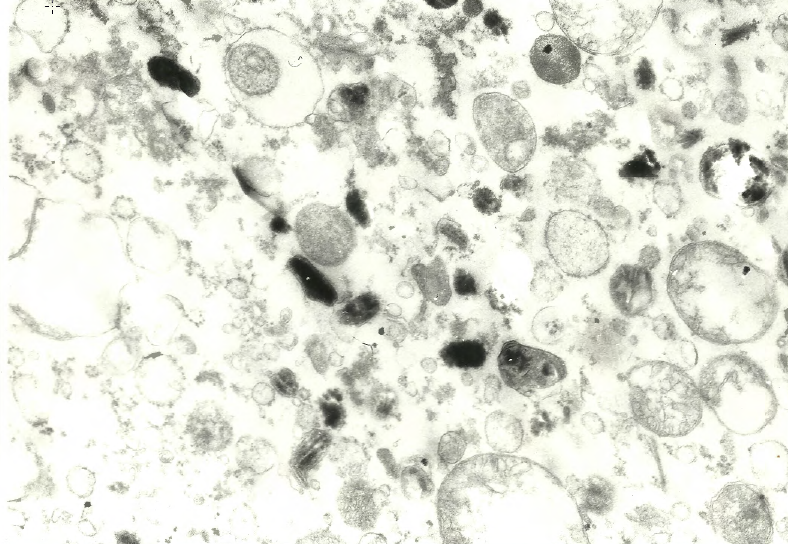
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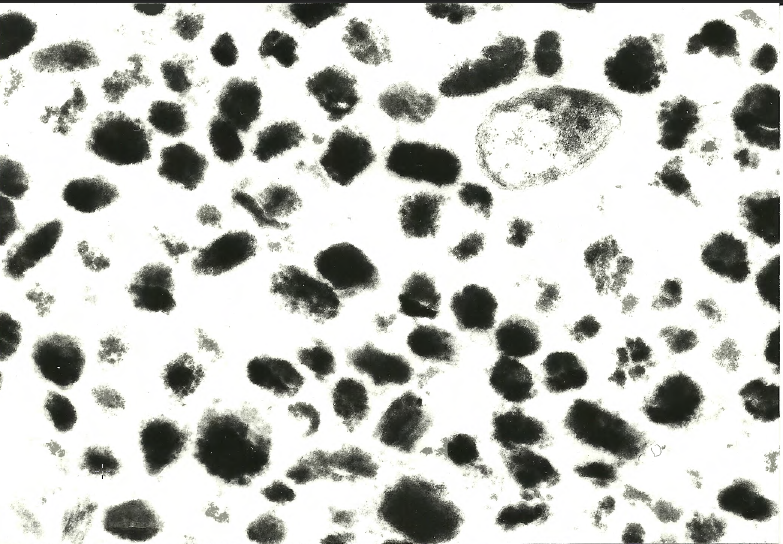
**Figure S18**: Photoconductivity of a synthetic DOPA melanin pellet measured in sandwich-electrode geometry and illuminated with white light. Two hydration states are shown (16.0% and 10.2%). The plots are normalised to the dark current and the wetter sample shows much higher photocurrent (as indicated by the Signal/Noise) and also larger negative photoconductivity associated with loss of water due to heating. The long rise-and-decay time constants are indicative of ionic effects. Reprinted with permission from Mostert et al. Appl. Phys. Letts 100, 093701. Copyright 2012. American Institute of Physics.



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**Figure S19**: Low contrast transmission electron micrographs (TEMs) showing the structure of synthetic DOPA melanin (a) [top] and natural bovine epithelium melanin (b) [bottom]. For both samples the indole quinone 2D sheets self assemble into a layered secondary structure. The stacking is held together by non-covalent interactions (Hunter-Sanders) and the inter-plane spacing is characteristic of hetero-aromatic systems at ~ 3.7 Å. The size of the sheets is limited by solvophobic effects and the system forms circular tertiary structures to minimise surface energy. Adapted from Watt *et al.,* Soft Matter 2009, 5, 3754 (with permission).





**Fig. S20** Electron micrographs of crude and purified melanosomal preparations. B16 mouse melanoma tumors were homogenized in an isotonic buffer (0.25 M sucrose in 10 mM phosphate buffer, pH 7.0). Nuclei and cell debris were removed by centrifugation and 700 x g, and a crude melanosomal preparation was obtained by centrifugation at 11,000 x g (20 min). The crude pellet was resuspended in isotonic buffer, and fractionated by ultracentrifugation in a discontinuous sucrose gradient, according to Menon and Haberman (1974). The pellets were stained with osmium tetroxide and observed with an electron microscope. Magnification: 20,000 x. A, crude melanosomal pellet. B, purified melanosome preparation.