Solid-state cross-polarization magic angle spinning 13C and 15N NMR characterization of Sepia melanin, Sepia melanin free acid and Human hair melanin in comparison with several mod...

ARTICLE *in* MAGNETIC RESONANCE IN CHEMISTRY · JUNE 2003

Impact Factor: 1.18 · DOI: 10.1002/mrc.1193

4 AUTHORS, INCLUDING:

Bhavin B. Adhyaru
Emory University
17 PUBLICATIONS **368** CITATIONS
SEE PROFILE

Clifford R. Bowers
University of Florida
94 PUBLICATIONS **1,867** CITATIONS
SEE PROFILE

MAGNETIC RESONANCE IN CHEMISTRY
Magn. Reson. Chem. 2003; **41**: 466–474
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/mrc.1193

Solid-state cross-polarization magic angle spinning ^{13}C and ^{15}N NMR characterization of *Sepia* melanin, *Sepia* melanin free acid and *Human hair* melanin in comparison with several model compounds

Bhavin B. Adhyaru, Novruz G. Akhmedov, Alan R. Katritzky and Clifford R. Bowers*

Department of Chemistry, University of Florida, P.O. Box 117200, Gainesville, Florida 32611-7200, USA

Received 8 November 2002; Revised 5 February 2003; Accepted 13 February 2003

This paper presents the high-resolution ^{13}C and ^{15}N cross-polarization magic angle spinning (CP/MAS) NMR spectra of three natural melanin solids: *Sepia officinalis* melanin, *Sepia officinalis* melanin free acid (MFA) and *Human hair* melanin. The functional group characterization of *Human hair* melanin by NMR is the first to date and the ^{13}C CP/MAS NMR spectra reported here show improved resolution of chemically inequivalent sites. The observed spectral regions of the solid melanin samples can be assigned to the postulated structural unit of the polymer chain of *Sepia* MFA derived from solution-state NMR studies. To assist in the assignment of functional groups in the spectra, the solid-state CP/MAS NMR spectra are compared with high-resolution ^{13}C and ^{15}N CP/MAS spectra of four model compounds, L-dopa, dopamine, 2-methoxycarbonyl-3-ethoxycarbonyl-4-methylpyrrole and ethyl 5,6-dimethoxyindole-2-carboxylate. To aid further in the assignment of protonated and non-protonated carbon atoms, CP contact time dependence and non-quaternary carbon suppression (NQS) experiments were performed on the melanin samples. The ^{15}N CP/MAS spectra of the melanin samples confirm the presence of indole and pyrrole units in the melanin polymer chain. The NMR peaks observed in all of the melanin samples are relatively broad, presumably owing to the presence of free radicals. Electron spin resonance (ESR) data shows that all three melanin samples contain localized free radicals (g = 2.007), with the *Sepia* melanin containing a 10-fold higher free radical density than *Human hair* melanin. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: solid-state NMR; ^{13}C CP/MAS; melanin; *Sepia*; *Human hair*

INTRODUCTION

Melanins are a class of pigments found in a diverse array of biological structures. While melanin is commonly associated with skin and hair color in humans, various types of melanins are also present in the inner ear, eyes, bird feathers, insect cuticles and the ink sac of *Sepia* cuttlefish. Research on melanins has revealed a variety of biological functions. For example, a special type of neuromelanin found within the substantia nigra of the human brain has been linked to neurodegenerative disorders (Parkinsonism, schizophrenia and Addison's disease), although the role of its involvement remains unclear.[1] Melanins also have importance in several nonbiological contexts. Insulating and semiconducting properties of melanins have been noted[2,3] and applications as ion-exchange resins[4] and redox polymers have been demonstrated.[5]

*Correspondence to: Clifford R. Bowers, Department of Chemistry, University of Florida, P.O. Box 117200, Gainesville, Florida 32611-7200, USA. E-mail: bowers@chem.ufl.edu
Contract/grant sponsor: NSF; Contract/grant number: CHE-9724635.

The suggested structures of the three predominant types of linkages between the 5,6-dihydroxyindole units found in melanins are shown in Fig. 1.[1]

The polymerization patterns resulting from these different types of linkages appear to govern functionality. A feature that has been observed by x-ray characterization is a 3.4 Å spacing corresponding to the parallel stacking of aromatic units in randomly oriented local domains.[2,6,7] X-ray analysis has also been used to examine the structure of *Sepia* melanin, L-dopa and tyrosine synthetic melanins.[8,9] These studies showed that melanins have short to intermediate range order.[9] Spectroscopic studies have been hampered by the insolubility and opacity of melanins. Structural characterizations have therefore turned to the analysis of the oxidative degradation products. Degradation studies on *Sepia* melanin show that it is a copolymer of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA)[10] as the principal constituents. Further oxidation of these units indicates that pyrrole mono-, di- and tricarboxylic acids could be present in the polymeric chain of the pigment. As an example, it was recently reported that *Sepia* melanin is a mixture

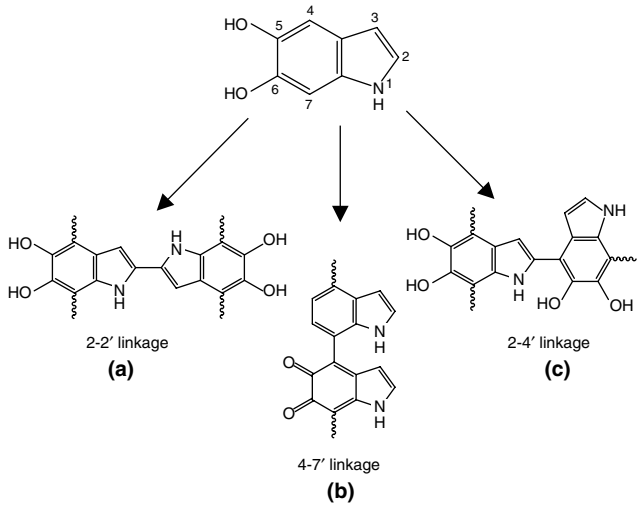

Figure 1. Melanin chain structure. Three types of linkages for 5,6-dihydroxyindole units are found within the melanin structures, as follows: (a) cross-linkages, links between pyrrole rings; (b) chain linkages, link through benzene-type rings alone, propagating a chain; (c) branching linkages, links between benzene and pyrrole rings, causing a branch from the main chain. The continuation of the polymer chain is indicated by wavy lines.

of oligomeric structures incorporating over 75% of DHICA-derived units and 20% of DHI-derived units, occurring for the most part in pyrrole carboxylic acid degraded form.[11]

Solution-state ^{1}H and ^{13}C NMR have also been utilized to study *Sepia* MFA in D_2O[12] and melanin isolated from the hydrolysis of black horsehair in D_2O–KOH.[13] Unfortunately, the spectra presented in these previous reports exhibited substantial line broadening, limiting their interpretation. Recently, the high-resolution ^{1}H spectra of the *Sepia* and *Sepia* melanin free acid (MFA), and *Human hair* melanin in D_2O (pH 10–11), have been reported, allowing quantification of the aromatic protons of the polymeric chain [the empirical formula for *Sepia* MFA normalized to one nitrogen was found to be $C_{7.35}H_{0.91}^{(vis)}H_{3.69}^{(invis)}NO_{3.60}$].[14]

Despite the great importance of this ubiquitous class of biopolymeric compounds, the rich diversity of structures of the melanins remains largely unexplored. The present work was motivated by the need to determine the structure formed from the postulated subunits [Figure 2(a), (**i**) or (**ii**)] of *Sepia* MFA.[14] In order to aid in the assignment of the ^{13}C cross-polarization magic angle spinning (CP/MAS) spectra of *Sepia* melanin, *Sepia* melanin free acid and *Human hair* melanin, we rely on ^{13}C and ^{15}N CP/MAS NMR spectra of the following four crystalline model compounds which are believed to have functional groups in common with the melanins: L-dopa (**1**), dopamine (**2**), ethyl 5,6-dimethoxyindole-2-carboxylate (**3**), and 2-methoxycarbonyl-3-ethoxycarbonyl-4-methylpyrrole (**4**). The structures of these model compounds are shown in Fig. 2(b). Dopamine and L-dopa were chosen because they are biological precursors in melanin biosynthesis. The spectral assignment of these compounds should provide insight into structural features present within the melanin compounds. Model compounds **3** and **4** were chosen based on the proposed structure of *Sepia* MFA from

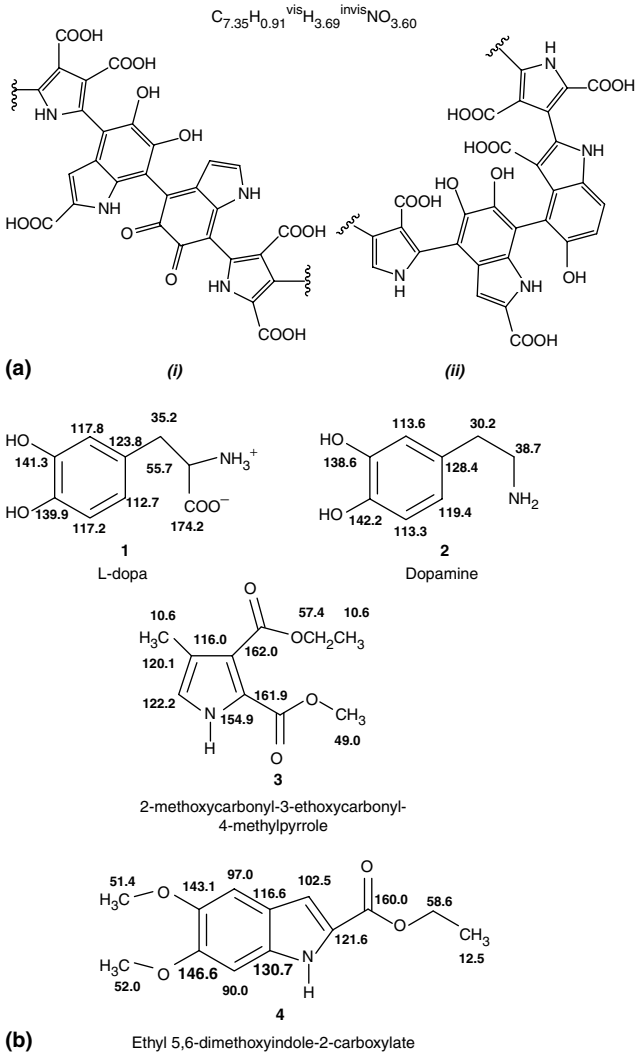

Figure 2. Structures of (a) proposed polymer chain structure for *Sepia* MFA and (b) model compounds. The model compounds contain functional groups similar to the melanin compounds. The carbon atoms on the model compounds are labeled with their respective chemical shifts from the ^{13}C CP/MAS NMR spectra.

solution-state studies.[14] In order to distinguish between protonated carbon atoms and quaternary carbon atoms, short CP contact time dependence and non-quaternary carbon suppression (NQS) experiments were performed.

EXPERIMENTAL

Materials

The *Sepia* melanin, *Sepia* MFA and *Human hair* melanin samples were obtained from Mel-Co (Orland, CA, USA). The model compounds were purchased from Sigma-Aldrich and used as received. The melanin samples were black powders (brown for *Human hair* melanin) and were used without further purification.

Solid-state NMR parameters

Solid-state ^{13}C CP/MAS NMR spectra were acquired on a Bruker Avance Spectrospin 400 MHz spectrometer operating

at 100.62 MHz using a triple resonance probe with a 4.0 mm rotor. Spectra were acquired at spinning rates of 8–10 kHz to ensure that all rotational sidebands would fall outside of the spectral region of interest. All ^{13}C NMR spectra are referenced to TMS. For CP/MAS experiments on the melanin samples, the CP parameters were optimized on a sample of dopamine. Typical experimental parameters were as follows: proton pulse duration, 4.0 μs; CP contact time, 1.2 ms; recycle delay, 5 s; spectral width, 50 kHz. High power proton decoupling was also employed in the CP/MAS experiments.

The efficiency of polarization transfer in the ^{13}C CP/MAS experiments depends on the relative strength of the ^{13}C–^{1}H dipolar interaction.[15–17] The CP contact time was varied to differentiate between protonated and quaternary carbon atoms in the melanin samples. The CP contact time dependence for various carbon atoms (C—H δ 30–60 versus C═O δ 180) for *Human hair* melanin is shown in Fig. 3. The plot shows that the ^{13}C intensity for protonated carbon atoms reaches a maximum at approximately 35 μs, at which point the intensity for the quaternary carbon atom is still fairly low.

In the NQS experiments, the magnetization of the non-quaternary carbons was dephased during a 43 μs pre-acquisition delay with no proton decoupling.[12] Approximately 3000–8000 transients were required to obtain melanin spectra with adequate signal-to-noise ratio. Prior to Fourier transformation, the time-domain signals were apodized using a line broadening of 100–200 Hz.

Natural abundance ^{15}N CP/MAS NMR spectra for the three melanin samples were also acquired at 40.56 MHz. The ^{15}N NMR spectra are referenced to NH_4OH. The spectral parameters are as follows: proton pulse duration, 4.0 μs; CP contact time, 1.2 ms; recycle delay, 5 s; spectral width, 50 kHz; number of transients, 20 000–30 000. The spectra were Fourier transformed using a 100 Hz line broadening.

ESR spectroscopy

Electron spin resonance (ESR) spectra were acquired on a Bruker (Billerica, MA, USA) ER 200D spectrometer modified with a digital signal channel and digital field controller. Data were collected using a U. S. EPR (Clarksville, MD, USA) SPEC300 data acquisition program and converted to ASCII format using a U. S. EPR EPRDAP data analysis program. All quantitative measurements were referenced to a DPPH (2,2-diphenyl-1-picrylhydrazyl) standard. The samples were weighed and placed in quartz tubes with the same diameter. The ESR spectra were recorded at 9.27 GHz at 295 K. The ESR signal was a single symmetric absorption with no hyperfine structure and the area was obtained by integration of the first derivative of the ESR signal. The free radical density (spins g^{-1}) for DPPH had been previously calibrated under similar experimental conditions. The number of free radicals for the melanin samples was determined by using a ratio of the area of the DDPH spectrum to the melanin spectrum corrected for receiver gain.

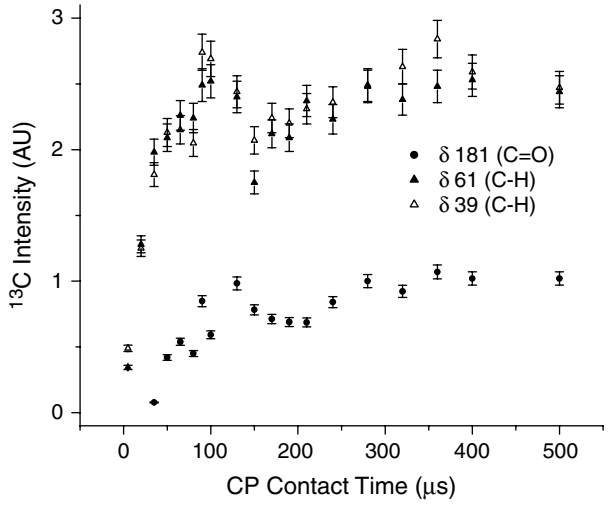

Figure 3. Plot of ^{13}C intensity versus CP contact time (μs) for *Human hair* melanin for different carbon types.

RESULTS AND DISCUSSION

^{13}C CP/MAS spectra of the model compounds

Figure 4(a)–(d) show the ^{13}C CP/MAS NMR spectra for the four model compounds. The representative NQS and CP/MAS spectra for **1** and **2** are shown in Fig. 5.

The correspondence between each carbon and its representative chemical shift is indicated on the molecular

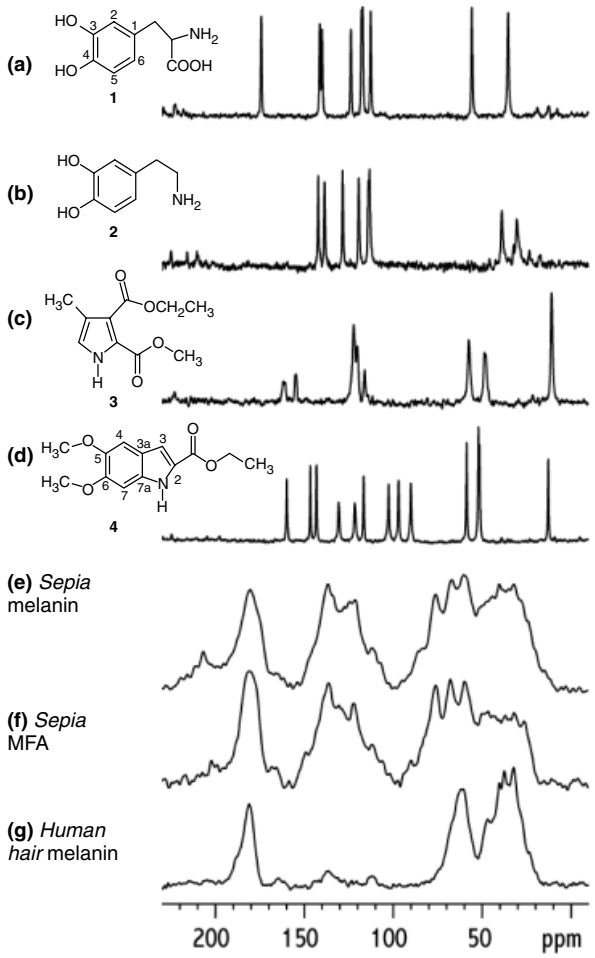

Figure 4. ^{13}C CP/MAS NMR spectra for the following model compounds and melanin samples: (a) **1**; (b) **2**; (c) **3**; (d) **4**; (e) *Sepia* melanin; (f) *Sepia* melanin free acid (MFA); (g) *Human hair* melanin.

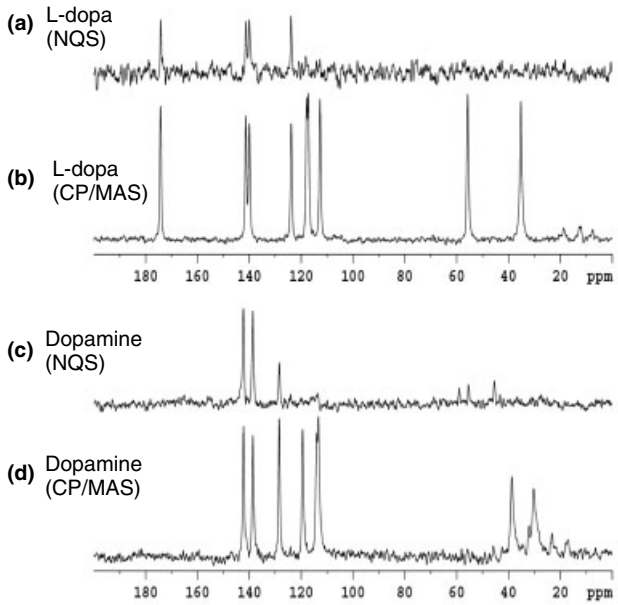

Figure 5. Comparison of NQS and CP/MAS spectra of **1** and **2**. (a) NQS spectrum of **1**; (b) CP/MAS spectrum of **1**; (c) NQS spectrum of **2**; (d) CP/MAS spectrum of **2**.

structure of **1**–**4** in Fig. 2(b). Unambiguous peak assignments of **1**–**4** provided from solid-state NMR spectra given in Fig. 2(b) are based on the solution-state NMR spectra (HETCOR and gHMBC). To illustrate the procedure used to assign the spectra of the model compounds, consider the assignments for **1** [Fig. 4(a)]. It is possible to identify three general regions attributable to aliphatic, aromatic and carbonyl functionalities. The aliphatic region consists of the CH_2 and CH carbons with chemical shifts of δ 35.2 and δ 55.7, respectively. Note that the CH carbon is deshielded by the attached functional groups. The aromatic and carbonyl regions can be assigned with the help of NQS spectral editing that suppresses all peaks due to non-quaternary carbons. In the NQS spectrum shown in Fig. 5(a), four resonances are observed. The resonance line with the highest shift of δ 174.2 represents the carboxyl carbon. The two resonances at δ 139.9 (C-2) and δ 141.3 (C-3) correspond to the *ipso* carbon atoms of the benzene ring. The last remaining *ipso* carbon, C-1, is assigned to the resonance at δ 123.8. The peaks (C-2 and C-5) that remain to be assigned for **1** are attributed to protonated CH carbon atoms of the benzene ring. The C-2 and C-5 carbons are expected to have similar chemical shifts, corresponding to δ 117.8 and δ 117.2, respectively. The resonance at δ 112.7 is assigned to C-6. Contour plots from HETCOR and gHMBC spectra of **2** and **4** are provided as representative examples. In **2**, the chemical shift values for C-2 (δ 113.6) and C-5 (δ 113.3) are easily assigned from the one-bond cross peaks (Figs 6 and 7). Spectra of the other model compounds were assigned in a similar manner.

Sepia melanin and *Sepia* melanin free acid

Figure 4(e)–(g) show the high-resolution ^{13}C CP/MAS NMR spectra for three types of melanins. The relative peak intensities in the ^{13}C CP/MAS NMR spectra of *Sepia* [Figs 4(e) and 8(a)] and *Sepia* MFA [Figs 4(f) and 8(b)] are indicative of the amount of indole and pyrrole units present, which appear to vary according to the source and isolation method of the melanin samples. There are some noticeable differences in comparison with previously reported ^{13}C CP/MAS spectra[12,18] of melanin samples (*Sepia* melanin and *Sepia* MFA), where an unambiguous assignment of the ^{13}C CP/MAS NMR peaks for protonated and quaternary carbon atoms was not possible owing to extensive broadening of the carbon signals. However, our functional group characterization of postulated units for *Sepia* MFA was facilitated by comparison with the spectra of model compounds **1**–**4** [Fig. 2(b)], short CP contact time experiments and NQS experiments. These spectra are shown in Fig. 8.

The characteristic spectral regions of all three melanin samples are summarized in Table 1. Three general types of resonances are identified, as follows: δ 10–95, aliphatic carbons, most likely due to proteinaceous material; δ 95–145, aromatic carbons, including indole or pyrrole type carbons within the polymer; and δ 165–200, carbonyl carbon atoms from amides, carboxylates, and quinones which may be associated with the melanin polymer as well as the proteinaceous material. The NQS experiments and CP contact time experiments allowed for assignment of δ 95–130 to protonated carbon atoms and δ 130–145 to quaternary carbon atoms in the melanin backbone.

On comparing the melanin spectra with those of several model compounds, the absorptions of the C═O group in the range δ 165–195 are close to carboxylate absorptions for the model compounds (**1**, **3** and **4**). The peaks at δ 90.0, 97.0 and 102.5 from the NMR spectrum of **4** are absent in the melanin spectra. This indicates that the melanin polymer chain is substituted at these positions [see suggested formula in Fig. 2(a)], hence the resonances for these carbon atoms

Table 1. Characteristic chemical shift regions of the ^{13}C CP/MAS NMR[a]

		Aromatic and Indolic		
Sample	Carbonyl	Cq	CH	Aliphatic
Sepia melanin	200–160	160–135	135–90	95–10
Sepia MFA	200–160	165–135	135–100	95–10
Human hair melanin	200–170	135–110 (Cq + CH)		90–50
				50–0

[a] Chemical shifts (δ) are reported in ppm, CH represents protonated carbons and Cq represents non-protonated carbons.

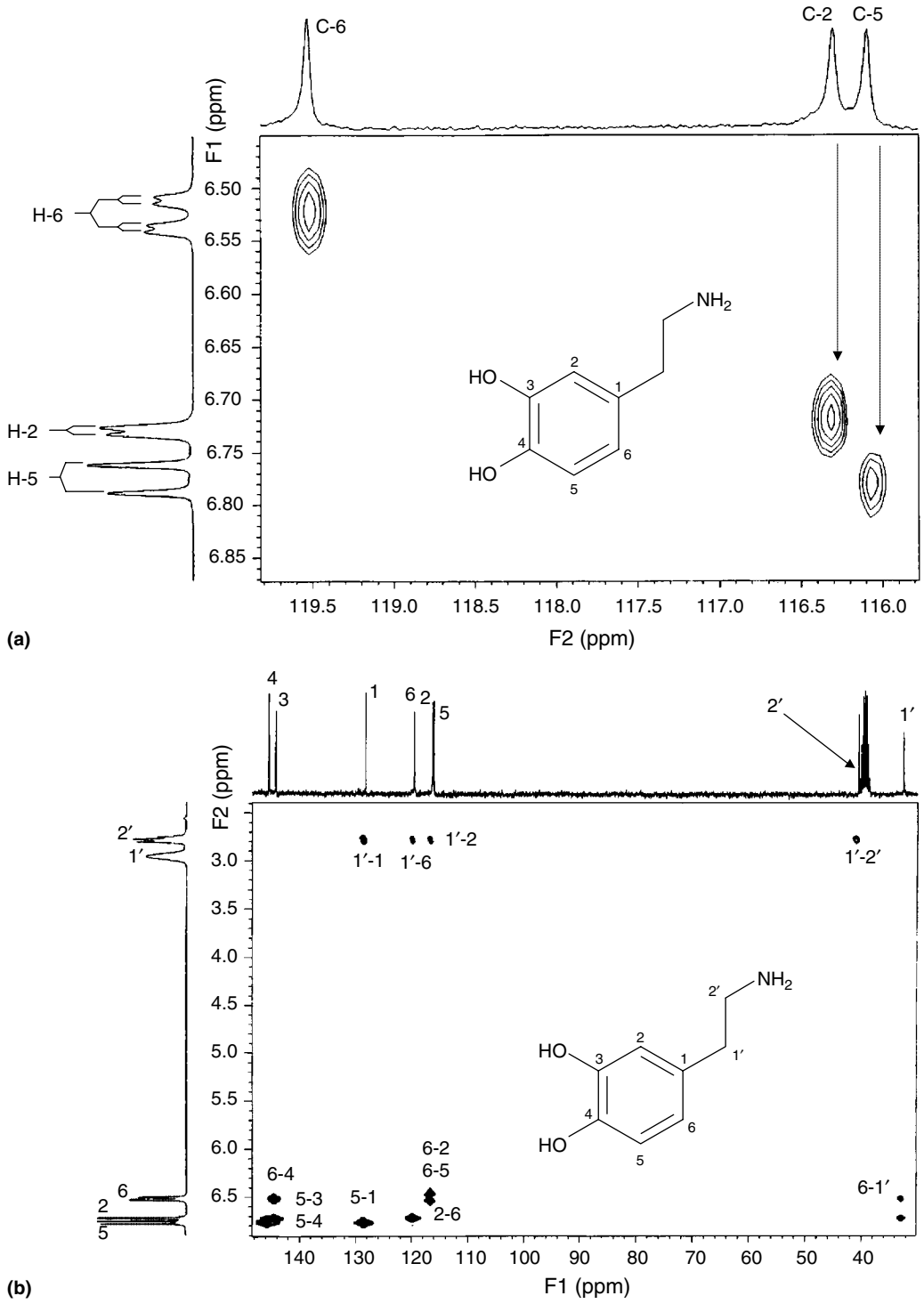

Figure 6. (a) HETCOR spectrum of **2** in DMSO-d_6 (aromatic part); (b) gHMBC spectrum of **2** in DMSO-d_6.

would exhibit higher chemical shifts. The presence of non-protonated quaternary carbons in the range δ 110–150 in the model compounds confirms the presence of indole and pyrrole units in the polymer chain of melanins.

A ^{13}C CP/MAS NMR spectrum was also acquired for the water-soluble derivative of *Sepia* melanin, *Sepia* MFA [Fig. 4(f)]. The observed chemical shift ranges for the solid-state NMR spectrum for *Sepia* MFA can roughly be determined from the suggested structural units of the polymer chain based on solution-state studies. The characteristic spectral regions δ 160–200 (carbonyl, carboxyl groups), δ 135–160 (oxygen-bearing carbon atoms from indole and pyrrole) and aliphatic (δ 10–95, attributable to protein material) correlate with the polymer chain in the postulated structural units of *Sepia* MFA. It is also apparent that the general features of the *Sepia* melanin spectrum are still present upon chemical modification, but the intensity of protonated carbon atoms in the aromatic region is lower relative to the *Sepia* melanin spectrum, as shown in the short contact time CP/MAS NMR spectrum [Fig. 8(b)]. Comparison of the melanin spectra with those of model compounds **1** and **2** reveals that the hydroxyl carbon peaks (δ 140–150) have

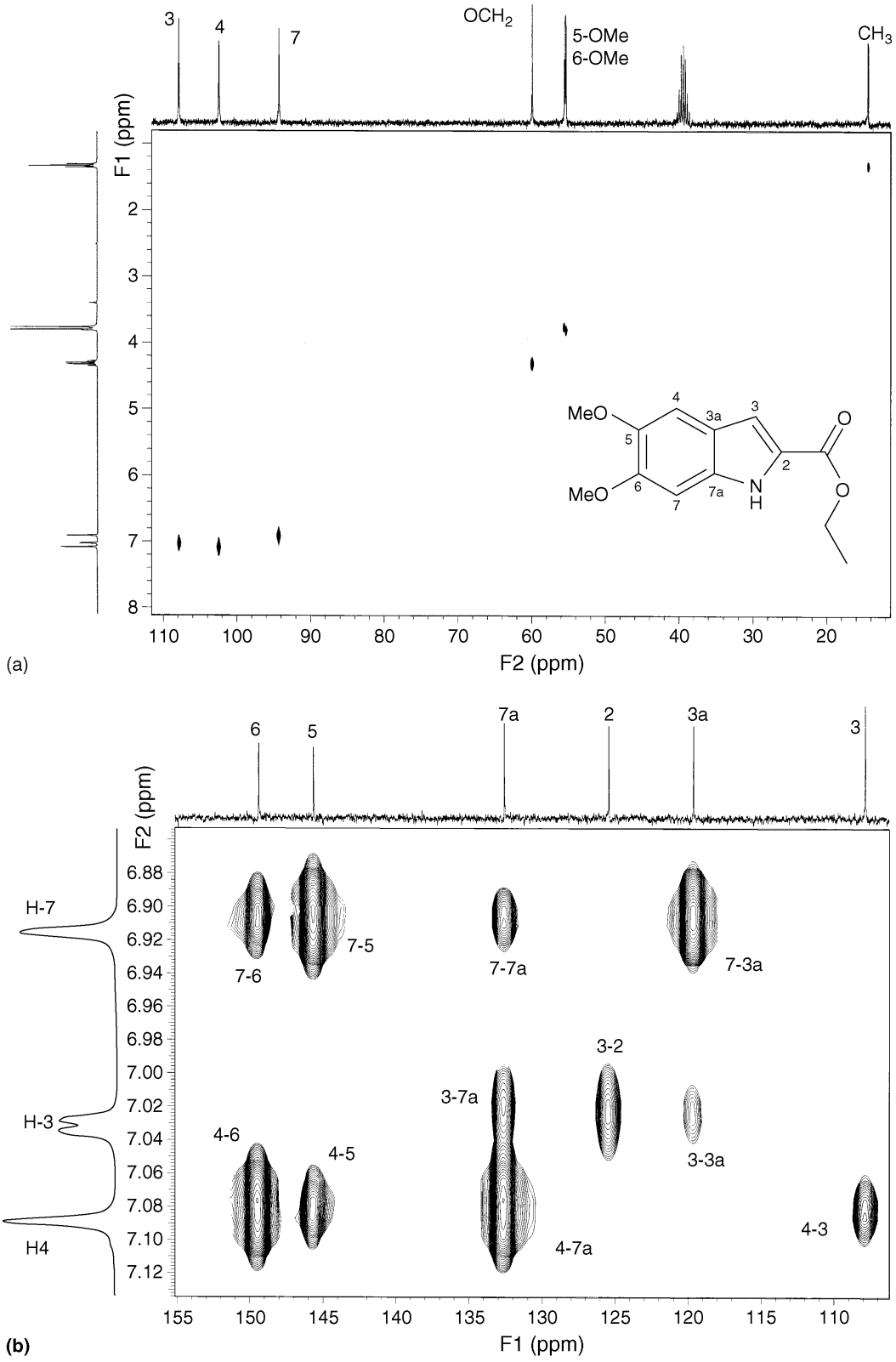

Figure 7. (a) HETCOR spectrum of **4** in DMSO-d_6; (b) gHMBC spectrum of **4** in DMSO-d_6 (aromatic part).

shifted to higher frequencies in *Sepia* melanin and *Sepia* MFA. This is probably due to oxidative phenol–quinone transformation of DHI (DHICA) units resulting in shifting C—O carbon atom resonances to higher frequencies (up to δ 160–180) or their degradation to pyrrolecarboxylic acids. In comparison with the previously reported spectra1,[12,19] our ^{13}C CP/MAS spectra exhibit improved chemical shift resolution, most notably in the δ 15–95 range (aliphatic region).[18]

Figure 9 compares the ^{15}N CP/MAS NMR spectra for all three melanin samples with those of model compounds **3** and **4**. The spectra for the melanin samples were acquired

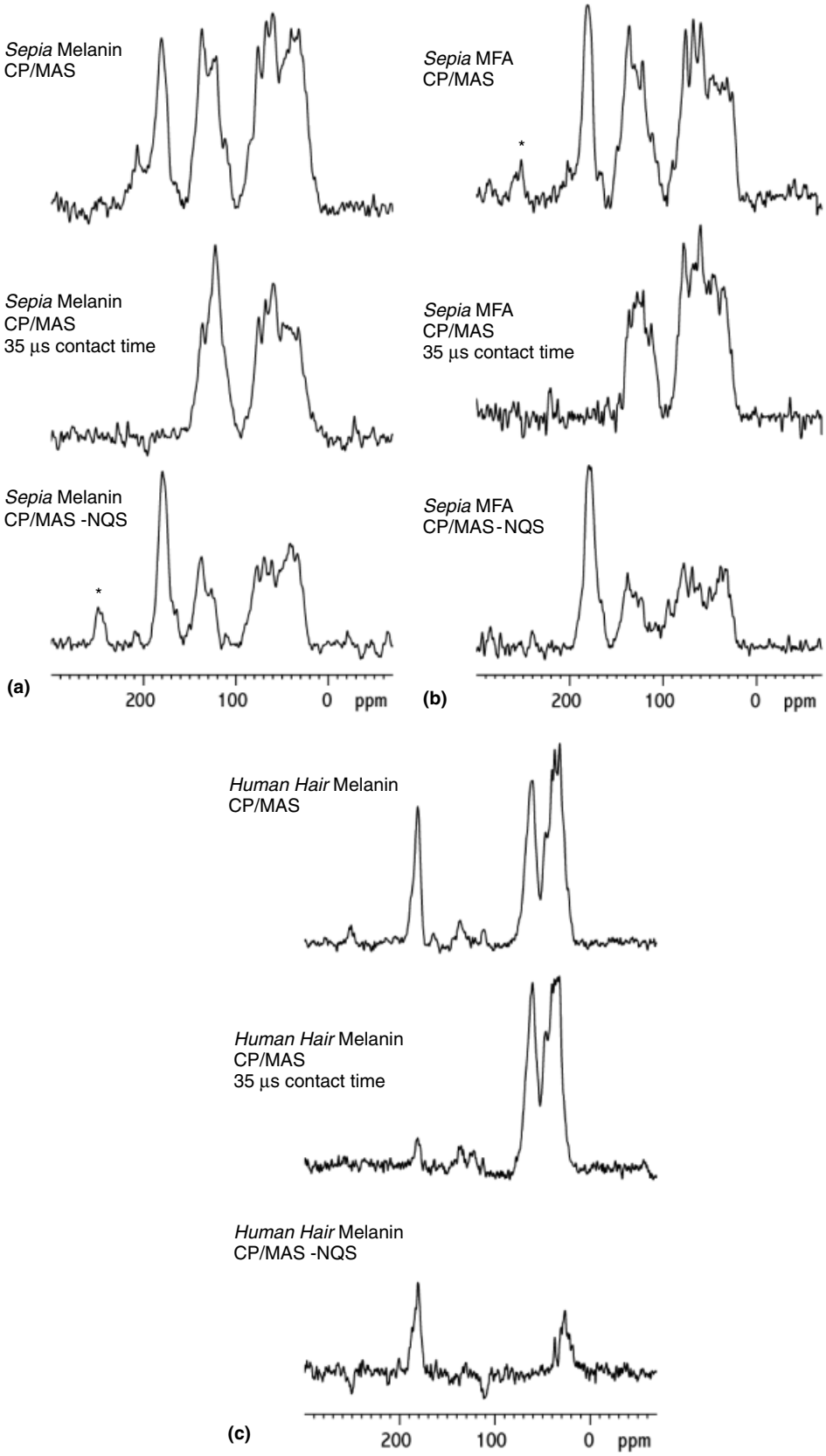

Figure 8. ^{13}C CP/MAS NMR spectra for (a) *Sepia* melanin, (b) *Sepia* melanin free acid and (c) *Human hair* melanin. In addition to the CP/MAS spectra, a short CP contact time experiment and a NQS experiment are included for each melanin sample. Spinning sidebands are marked with an asterisk.

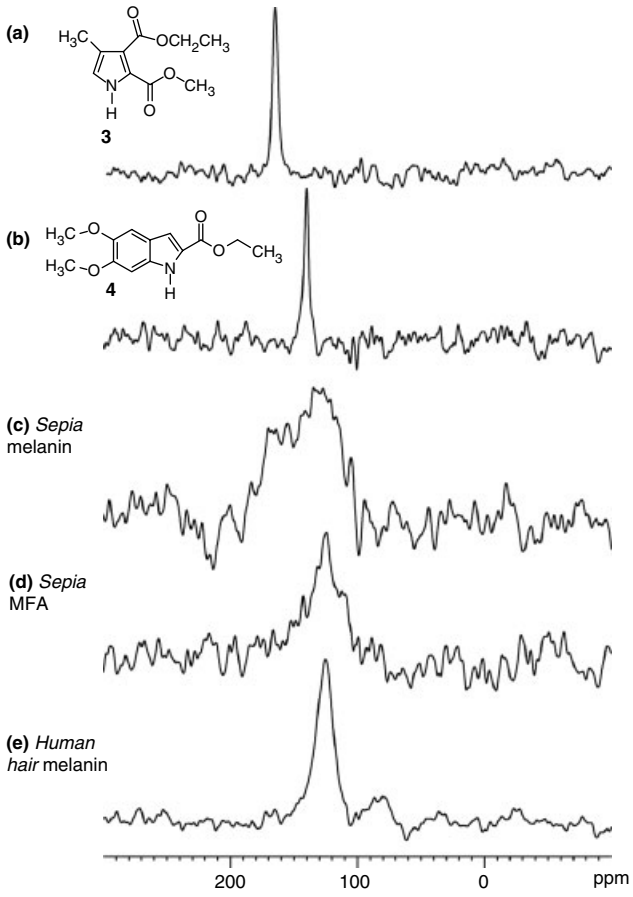

Figure 9. Nitrogen-15 CP/MAS NMR spectra for the following model compounds and melanin samples: (a) model compound **3**; (b) model compound **4**; (c) *Sepia* melanin; (d) *Sepia* melanin free acid (MFA); (e) *Human hair* melanin.

under similar experimental conditions. The *Sepia* melanin spectrum exhibits a broad peak from approximately δ 110 to 180, indicating a variation in the linkages between indolic and pyrrolic groups in the polymer structure. The ^{15}N CP/MAS spectrum for the *Sepia* MFA is very different, exhibiting a less intense (relative to the *Sepia* melanin) but much narrower peak in the range δ 110–140. This may be indicative of modification of nitrogen-containing groups that occurs upon oxidation of the *Sepia* melanin.

Human hair melanin

Human hair melanin consists of a melanin backbone with attached peptide fragments.[20] Amino acids account for 65.8% of the mass composition of the material (compared with 6.17% found in the *Sepia* melanin sample); of this, 60.7% of the amino acid composition contain an aliphatic group, 8.8% contains aromatic groups (tyrosine, phenylalanine, tryptophan, and histidine) and the remaining 30.5% consists of polycarboxylic amino acids (aspartic and glutamic acid).[20] To our knowledge, the ^{13}C CP/MAS spectrum of *Human hair* melanin has not been reported previously. We present ^{13}C CP/MAS NMR spectra of *Human hair* melanin in Figs 4(g) and 8(c). The spectrum exhibits the three characteristic regions described above, but in comparison with the spectra of the other two melanin compounds shown in Figs 4(e) and (f), the ratio of the aliphatic to aromatic signals is substantially larger for the *Human hair* melanin sample. This is presumably attributable to the greater percentage of aliphatic amino acids present in the *Human hair* melanin sample compared with *Sepia* melanin. The absorption in the aromatic region (δ 110–140) is tentatively assigned to the melanin backbone, although there could be contributions to this broad signal from the aromatic amino acids. The region δ 160–185 is assigned to carboxyl and carbonyl carbon atoms, but again, there is also a contribution from the polypeptide fragments in this part of the spectrum. The solution-state ^{1}H NMR spectrum also exhibits broad resonances in the aromatic region and also shows a much larger ratio of aliphatic to aromatic signals.[14] The difficulty in identifying and quantifying the aromatic protons might be avoided if the melanin polymer could be separated from the associated polypeptide fragments, but there have been no reports of accomplishing this without disruption of the melanin polymer structure. The ^{15}N CP/MAS spectrum of *Human hair* [Fig. 9(e)] shows a single broad (compared with spectra for **3** and **4**) resonance at δ 138, which is within the indole/pyrrole chemical shift region. Interestingly, the *Sepia* MFA and *Human hair* melanin spectra display peaks at similar chemical shifts, though the signal-to-noise ratio in the *Sepia* MFA spectrum is much smaller.

The ^{13}C CP/MAS spectrum of *Human hair* melanin exhibits substantial differences in comparison with the *Sepia* and *Sepia* MFA spectra. The intensity of peaks in the range δ 110–150 is much lower for the aliphatic regions and carboxyl region peaks. This could be explained by the presence of more non-protonated carbon resonances from the indole and pyrrole units of the polymer chain melanins. Chemical shifts in the ranges δ 105–120 and δ 130–145 are very close to chemical shifts for the oxygen-bearing carbon and protonated carbons found in model compounds **1**, **2** and **4** (Fig. 4).

The ^{13}C CP/MAS spectra of melanins exhibited broad peaks suggesting the presence of paramagnetic free radical centers. ESR spectroscopy has been used to study electron paramagnetism in synthetic Dopa melanin and natural melanoma melanin.[19] We employed ESR to quantify the density of free radicals in our melanin samples. The ESR signals of the *Sepia* melanin and *Human hair* melanin consist of a single absorption line with no noticeable hyperfine structure. We found that the *Sepia* melanin contained $(2 \pm 4) \times 10^{17}$ free radicals g^{-1}, whereas the *Human hair* melanin sample contained only $(2 \pm 3) \times 10^{16}$ free radicals g^{-1}. This could explain why somewhat narrower ^{13}C resonances were obtained in the case of *Human hair* melanin in comparison with the *Sepia* melanin. Literature ESR data on melanin obtained from Japanese black hair[21] also shows a single absorption line; however, the free radical concentration differed and was found to be 3.6×10^{18} free radicals g^{-1}. The difference in free radical content can be attributed to several factors, such as water content (known to reduce the ESR signal) and pH (increased acidity reduces the signal, whereas increased basicity increases the ESR signal).[21]

CONCLUSION

We have presented the solid-state ^{13}C and ^{15}N CP/MAS NMR spectra of *Sepia* melanin, *Sepia* melanin free acid and

Human hair melanin. A functional group characterization was performed by assignment of the spectra for the melanin samples using established carbon resonance chemical shifts for several model compounds. The ^{13}C and ^{15}N MAS NMR characterization of *Human hair* melanin is the first to date. The ^{15}N spectrum for *Human hair* melanin shows an intense peak (relative to the *Sepia* melanin spectrum) at δ 138 which probably indicates the presence of indole groups from the melanin. Finally, the ESR data show that the *Human hair* melanin sample has an order of magnitude lower free radical density than the *Sepia* melanin sample, a finding that could explain the greater line broadening in the latter.

Acknowledgments

The authors thank Jonathan Woodward for assistance in determining the free radical content of the melanin samples by ESR. Support for this work was obtained from NSF Major Research Instrumentation grant CHE-9724635.

REFERENCES

1. Aime S, Fasano M, Bergamasco B, Lopiano L, Quattrocolo G. *Adv Neurol.* 1996; **69**: 263.
2. Swan GA. *Fortschr. Chem. Org. Naturst.* 1974; **31**: 521.
3. Pullman A, Pullman B. *Biochim. Biophys. Acta* 1961; **54**: 384.
4. Lindquist NG. *Uppsala J. Med. Sci.* 1986; **91**: 283.
5. Sarna T, Fronscisz W, Hyde JS. *Arch. Biochem. Biophys.* 1980; **202**: 304.
6. Nicolaus RA. *Melanins.* Hermann: Paris, 1968.
7. Blois MS, Smith KC. *Photochem. Photobiol. Rev.* 1978; **3**: 115.
8. Cheng J, Moss SC, Eisner M, Zschock P. *Pigm. Cell Res.* 1994; **7**: 255.
9. Cheng J, Moss SC, Eisner M. *Pigm. Cell Res.* 1994; **7**: 263.
10. Nicolaus RA, Piattelli M, Fattorosso E. *Tetrahedron* 1964; **20**: 1163.
11. Pezzella A, D'Ischia M, Napollitano A, Palumbo A, Prota G. *Tetrahedron* 1997; **53**: 8281.
12. Aime S, Fasano M, Terreno E, Groombridge CJ. *Pigm. Cell Res.* 1991; **4**: 216.
13. Golounin AV. *Russ. J. Appl. Chem.* 1996; **69**: 103.
14. Katritzky AR, Akhmedov NG, Denisenko SN, Denisko OV. *Pigm. Cell Res.* 2002; **15**: 93.
15. Herve M, Hirschinger J, Granger P, Gilard P, Goetz N. *J. Chim. Phys.* 1994; **91**: 881.
16. Peter MG, Forster H. *Angew. Chem. Int. Ed. Engl.* 1989; **28**: 741.
17. Duff GA, Robers JE, Foster N. *Biochemistry* 1998; **27**: 7112.
18. Amie S, Fasano M, Croombridge C. *Gazz. Chim. Ital.* 1990; **120**: 663.
19. Mason HS, Ingram DJE, Allen B. *Arch. Biochem. Biophys.* 1960; **86**: 225.
20. *Amino Acid Composition of Sepia Melanin, Sepia Melanin Free Acid, and Human hair Melanin.* Mel Co., Orland, CA, internal unpublished communication, 2001.
21. Arnaud R, Perbet G, Deflandre A. *Photochem. Photobiol.* 1983; **38**: 161.