Pergamon

Tetrahedron, Vol. 52, No. 23, pp. 7913-7920, 1996
Copyright © 1996 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0040-4020/96 $15.00 + 0.00

PII: S0040-4020(96)00362-6

Oxidative Polymerisation of 5,6-Dihydroxyindole-2-carboxylic Acid to Melanin: A New Insight

Alessandro Pezzella, Alessandra Napolitano, Marco d'Ischia and Giuseppe Prota*

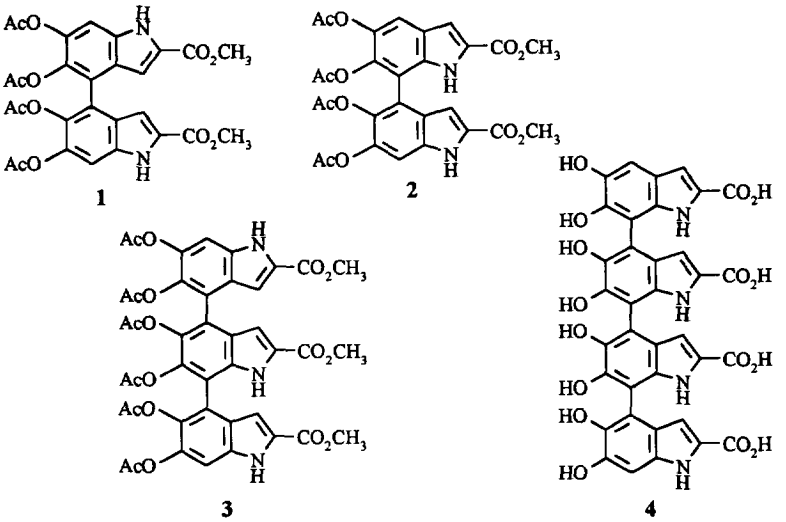
Department of Organic and Biological Chemistry, University of Naples Federico II,
Via Mezzocannone 16, I-80134 Naples, Italy.

Abstract. Previous studies on the oxidative polymerisation of 5,6-dihydroxyindole-2-carboxylic acid, a key intermediate in the biosynthesis of eumelanins, had delineated a reaction pathway involving mainly repeated coupling of the indole units through the 4- and 7- positions. Using an improved HPLC methodology for the direct analysis of oligomer intermediates, we have now obtained evidence for a more complex mode of polymerisation, involving formation, besides the 4,4' and 4,7' coupled dimers **5** and **6**, of three new dimers, which have been isolated and identified as the 3,4'-, 3,7'- and 7,7'-biindolyls **7-9**. The observed implication of the 3-position is unprecedented in the oxidative polymerisation of 5,6-dihydroxyindoles and yields important clues for future studies aimed at elucidating the chemical constitution of natural and synthetic eumelanins.
Copyright © 1996 Elsevier Science Ltd

Progress in the unravelling of the structure of eumelanins, the main determinants of dark skin and hair colorations, has relied to a considerable extent on an understanding of the mechanism of polymerisation of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid.[1] In spite of vigorous and unabated interest over the past 50 years, realisation of this goal has been regarded until not too long ago as a virtually impossible task, the main difficulties lying in the intrinsic complexity of the reaction patterns coupled with the elusive character of the intermediate products.

A fundamental breakthrough came when a specific procedure was devised that allowed for the first time the isolation of sufficient amounts of oligomer intermediates for chemical investigation.[2] This involved reduction of the oxidation mixture with sodium dithionite and subsequent acetylation of the ethyl acetate extractable fraction with acetic anhydride. In this way, the basic pattern of polymerisation of 5,6-dihydroxyindole was disclosed, and was shown to consist mainly of sequential coupling processes through 2,4' and 2,7' bonds.[3-6]

By a similar approach, it was found that 5,6-dihydroxyindole-2-carboxylic acid tends to polymerise *via* the 4- and 7-positions, as reflected by the structures of the dimers **1** and **2**, and of the trimer **3**, obtained as the acetate and methyl ester derivatives.[6-8] Notably, the linear assemblage of the indole units indicated by structures **1-3** turned out to be in fairly good agreement with a traditionally reported model for 5,6-dihydroxyindole-2-carboxylic acid polymers. This had been deduced largely on the basis of theoretical considerations,[9,10] and was lent support by the presumptive isolation of the oligomer **4** from the *tapetum lucidum* of the catfish.[11] The lack of reactivity of the 3-position was generally ascribed to a deactivating effect imparted by the adjacent carboxy group.



In furthering our programme on the structure of eumelanins, we happened to re-examine the oxidation of 5,6-dihydroxyindole-2-carboxylic acid. Quite unexpectedly, application of a newly developed methodology for the direct analysis of the oligomer intermediates unveiled the formation of a reproducible set of products whose elutographic properties were suggestive of oligomers at a low degree of polymerisation. Such pattern of products, which is apparent from the typical HPLC elutogram shown in Figure 1, was evidently difficult to rationalise in terms of structures related solely to **1-3**.

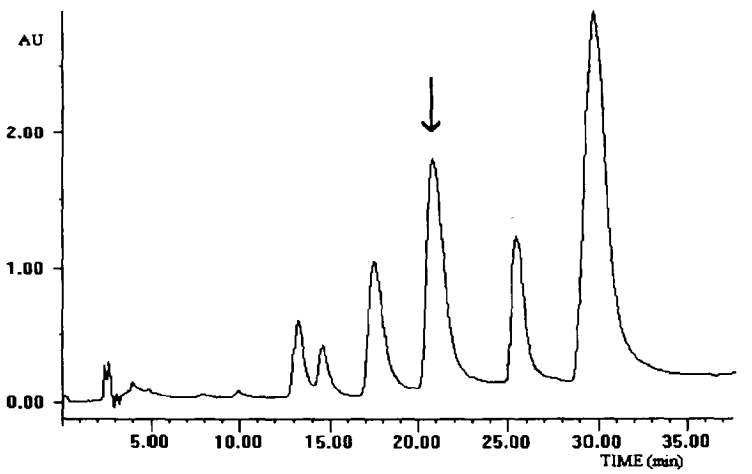


Figure 1. HPLC elutographic profile of the oligomer products obtained by cupric ions-promoted autoxidation of 5,6-dihydroxyindole-2-carboxylic acid in 0.5 M Tris buffer, pH 7.5. The arrow indicates unreacted 5,6-dihydroxyindole-2-carboxylic acid.

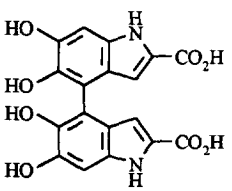
Qualitatively similar elution profiles were obtained when the reaction was carried out using different enzymatic and chemical oxidising systems, such as tyrosinase, peroxidase/hydrogen peroxide, potassium ferricyanide and oxygen/ cupric ions. Insight into the structure of these products was thus gained by appropriate scale up of the experimental protocol and optimisation of the HPLC elution system for preparative purposes.

Under the majority of the oxidation conditions examined, the dominant product proved to be the compound eluting at about 30 min in the chromatogram shown in Figure 1. This was isolated by preparative HPLC and was identified as the 4,4' dimer **5** by straightforward spectral analysis.[12] The proposed assignment was secured by comparison of the spectral and chromatographic properties of its acetyl, methyl ester derivative with those of an authentic sample of **1**.[6,7]

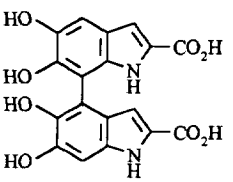
Another relatively more abundant product was the compound eluted at 26 min in the HPLC elutogram. This displayed a pseudomolecular ion peak ($M+H^+$) at m/z = 385 in the FAB-MS spectrum, indicating a dimer. From analysis of the proton and carbon resonance spectra, in which one 4- and one 7-position of the indole rings were clearly engaged in a carbon-carbon bond, the compound was readily assigned structure **6**. This was confirmed by comparison of the properties of the acetyl, methyl ester derivative with those of the dimer **2**.[7]

The identification of dimers **5** and **6** was essentially confirmatory of a preferred mode of polymerisation of 5,6-dihydroxyindole-2-carboxylic acid *via* the 4- and 7-positions. The attention was then shifted to the other major components of the oxidation mixture which, though not so abundant as **5**, provided in most cases a substantial contribution to the overall mass balance at the early oxidation stage.

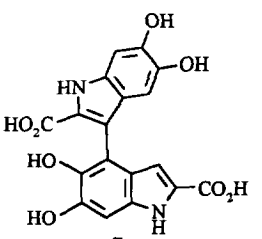
A significant peak in some of chromatograms examined was due to a compound eluting at 18 min, i.e. at a faster time than that of the parent indole. Spectral analysis (FAB-MS and ^{1}H-NMR) provided evidence for a dimer in which, notably, an H-3 proton was apparently missing. Scrutiny of the carbon resonance pattern revealed two CH signals at δ 95.53 and 96.84, which exhibited one-bond correlation with two virtually overlapped singlets in the proton spectrum (δ 6.80 and 6.81), indicating two unsubstituted C-7 carbons.



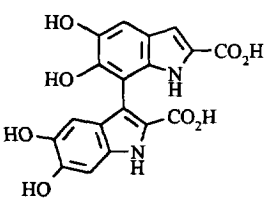
5



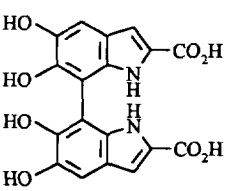
6



7



8



9

These and other data, in view of the lack of symmetry of the molecule, could only be accommodated by the structure of the 3,4'-linked dimer **7**. A notable characteristic of the proton spectrum is the marked high-field shift of the 4 and 3' protons, which probably occupy a shielding environment above the attached indole ring.

An additional feature of most HPLC elutograms was the presence of two peaks which were relatively less retained on reverse phase, being eluted at about 13 and 15 min. After careful selection of elutographic condition and repeated preparative HPLC, the two compounds were eventually isolated in pure form and shown to be dimers by mass spectrometric analysis.

The compound eluted at 15 min was readily identified as the 3,7'-coupled dimer **8**, on the basis of the lack of one H-7 and one H-3 signal in the proton spectrum and a consistent pattern of resonances in the ^{13}C-NMR spectrum. The other dimer displayed NMR features that were indicative of a symmetric mode of coupling of the indole units. Of the two possible options available, i.e. a 3,3' and a 7,7'-coupled dimer, the latter was clearly favoured on the basis of the chemical shift of the proton and carbon resonances of the unsubstituted ring positions. Further evidence for such a mode of coupling (structure **9**) was provided by a relatively shielded quaternary carbon at δ 104.46, typical of C-7-linked dimers.

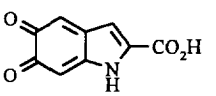
In subsequent experiments, the variations of the product distribution with the oxidation conditions and other experimental parameters were examined. The Table shows the relative yields of the various dimers determined in the early stages of the process, when no more than half of the starting indole was consumed, to ensure limited oxidation of the oligomer products. It appears that tyrosinase, peroxidase/hydrogen peroxide and ferricyanide induce a dominant reaction path *via* 4,4'- and 4,7'-couplings, with a modest contribution of the 7,7'-coupling. Cupric ion-promoted aerial oxidations, on the other hand, cause the alternate modes of dimerisation, through the 3-position, to become competitive. Of particular interest was the marked variation in the product distribution with the cupric ion-to-substrate ratio and the pH of the buffer. In all cases examined the product distribution was found to remain constant in the early stages of the oxidation.

Table. Relative Percent Yields of Dimers **5-9** Obtained by Oxidation of 5,6-Dihydroxyindole-2-carboxylic Acid under Different Conditions.

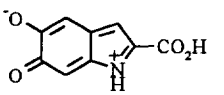
Compound	Yield (%)[a]					
	Tyr/O_2	*Perox/H_2O_2*	*$K_3Fe(CN)_6$*	*Cu^{2+}/O_2*[b] *(0.5:1)*[d]	*Cu^{2+}/O_2*[c] *(1:1)*[d]	*Cu^{2+}/O_2*[c] *(2:1)*[d]
5	60	40	60	33	63	61
6	30	41	36	29	16	10
7	2	11	n.d.	5	10	14
8	1	n.d.	n.d.	n.d	2	5
9	7	8	4	33	9	10

[a] determined by HPLC, mean of two experiments, expressed as percent of the total yields of **5-9**.
[b] in 0.5 M Tris buffer, pH 6.5. [c] in 0.5 M Tris buffer, pH 7.5.[d] cupric ions/indole molar ratio.

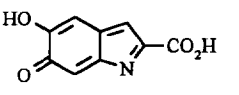
Apart from the interest relating to the structure of the eumelanin pigment backbone, the isolation of the 3-linked dimers **7** and **8** overthrows a basic tenet in traditional views on the polymerisation of 5,6-dihydroxyindole-2-carboxylic acid, and raises intriguing mechanistic points. If it is allowed that coupling follows from indole-quinone interactions, one could then guess that attack to the 3-position is largely a reflection of a significant electron deficiency at that site in a quinonoid intermediate. This, however, is difficult to rationalise in terms of structures like **10**, commonly reported for 5,6-indolequinone-2-carboxylic acid. A plausible explanation could envisage either a significant contribution of structure **11** to the resonance hybrid, or a tautomerisation step leading from **10** to the quinone methide **12**.



10



11



12

While the former option relies on feeble arguments, the latter would be compatible with a stabilising effect of the carboxy group, ensuring that the elusive quinone **10** survives enough to be converted at least in part into **12**. It may be relevant to notice that the formation of quinone methide tautomers in the oxidative polymerisation of 5,6-dihydroxyindoles had been inferred on the basis of pulse radiolytic experiments.[13] In this context, the observed effect of metal ions and alkaline pHs, which enhance coupling at the 3-position, may be rationalised in terms of an alteration of the equilibrium between **10** and **12** in favour of the latter species. Other factors, however, including formation of metal complexes with the *o*-diphenol moiety of the indole and variations in the rate of the oxidation process may as well account for the observed variations in the distribution of dimers **5-9**.

As a concluding remark, we wish to emphasise that dimers **5-9** are the first 5,6-dihydroxyindole oligomers to be isolated without prior derivatisation. This represents an important methodological achievement that has permitted insight into hitherto unrecognised facets of the oxidation chemistry of the 5,6-dihydroxyindole system, and that may open promising perspectives for a deeper understanding of the structure of eumelanins.

ACKNOWLEDGEMENTS

This work was supported by grants from Consiglio Nazionale delle Ricerche (Rome), Ministero dell'Università e della Ricerca Scientifica e Tecnologica (Rome) and the Lawrence M. Gelb Research Foundation (Stamford, CT, USA). Mass spectra were performed by the Servizio di Spettrometria di Massa del CNR e dell'Università di Napoli. The assistance of the staff is gratefully acknowledged. We thank Miss Silvana Corsani for technical assistance.

EXPERIMENTAL

Melting points were recorded on a Kofler hot-stage apparatus and are uncorrected. UV spectra were performed with a Perkin-Elmer Lambda 7 spectrophotometer. ^{1}H-NMR (400.1 and 270.1 MHz) and ^{13}C-NMR (100.6 and 67.9 MHz) spectra were carried out on Bruker WM 400 and AC 270 spectrometers. Tetramethylsilane was used as reference standard. 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. Experiments were recorded with 128 x 2048 matrix sizes. High resolution FAB-MS spectra (positive mode) were determined on a VG model ZAB-2F SE spectrometer. A mixture of glycerol and thioglycerol was used as the matrix. 5,6-Dihydroxyindole-2-carboxylic acid was prepared by a literature procedure.[14] 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% (stabilised), potassium ferricyanide, cupric sulphate pentahydrate and all other chemicals were purchased from Aldrich. Glass distilled, deionised water was used for preparation of all solutions. HPLC analyses were carried out on a Gilson apparatus equipped with a Gilson mod. 117 UV detector set at 280 nm. RP18 Spherisorb S5ODS2 (4.0 x 250 mm, Phase Separation Ltd.) or Econosil C-18 10U (22 x 250 mm, Alltech) columns were used for analytical and preparative purposes, with flow rates of 1 mL/min and 10 mL/min, respectively.

Oxidation of 5,6-dihydroxyindole-2-carboxylic acid

A solution of the indole (26 μmol) in phosphate buffer 0.1 M pH 7.0 (3 ml) was treated with a) tyrosinase (400 units) under a stream of oxygen, b) peroxidase (400 units)/ 0.3 % hydrogen peroxide (220 μl), c) potassium ferricyanide (26 μmol) or d) varying amounts of cupric sulphate (13, 26 or 52 μmol). In the latter case the buffer system was 0.5 M Tris, at pH 7.5 in the experiments at 2:1, and 1:1 cupric ions/indole molar ratio, and pH 6.5 in the experiments at 0.5:1 cupric ions/indole molar ratio. Enzymatic oxidations were performed in a thermostatic bath regulated at 25°C. After 10 min in the tyrosinase catalysed oxidation, and 1 min in all other cases, the reaction was stopped 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 analysed by HPLC using mixed 0.05 M ammonium citrate-0.4 M ammonium formate, pH 2.5 containing 6% acetonitrile as the mobile phase.

Isolation of dimers ***5-9***

For the isolation of dimers **5-9**, a solution of 5,6-dihydroxyindole-2-carboxylic acid (500 mg) in 0.5 M Tris buffer, pH 7.5 (300 ml), was saturated with oxygen gas and then treated with cupric sulphate (630 mg) in water (2 ml) under vigorous stirring. After about 1 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 200 mg) was taken up in DMSO (1 ml) and chromatographed on preparative HPLC, using 0.4 M formic acid as the mobile phase. The peaks eluted at 30, 35, 45, 60, and 70 min were collected and carefully evaporated to dryness at room temperature to give pure dimers **8** (7 mg), **9** (15 mg), **7** (20 mg), **6** (40 mg) ,**5** (100 mg) in

the order. Aliquots of compounds **5** and **6** were subjected to esterification and acetylation as previously reported[7] to give products identical in all respects to authentic **1** and **2**.

2,2'-Dicarboxy-5,5',6,6'-tetrahydroxy-4,4'-biindolyl (5): grey solid; UV: λ_{max} (EtOH) 328 nm (log ε 4.35); HR FAB-MS 385.0680 $(M+H)^+$ (calc. for $C_{18}H_{13}N_2O_8$: 385.0672); ^{1}H-NMR (DMSO-d_6): δ 6.31 (1Hx2, s, H-3 and H-3'), 6.87 (1Hx2, s, H-7 and H-7'), 11.14 (1Hx2, bs, NH and NH'). ^{13}C-NMR (DMSO-d_6): δ 96.01 (CH, C-7 and C-7'), 108.12 (CH, C-3 and C-3'), 114.42 (C, C-4 and C-4'), 120.43 (C, C-9 and C-9'), 125.24 (C, C-2 and C-2'), 132.17 (C, C-8 and C-8'), 139.07 (C, C-5 and C-5'), 146.34 (C, C-6 and C-6'), 162.85 (C, carboxy groups).

2,2'-Dicarboxy-5,5',6,6'-tetrahydroxy-4,7'-biindolyl (6): grey solid; UV: λ_{max} (EtOH) 327 nm (log ε 4.30); HR FAB-MS 385.0663 $(M+H)^+$ (calc. for $C_{18}H_{13}N_2O_8$: 385.0672);^{1}H-NMR (DMSO-d_6): δ 6.30 (1H, d, J= 1.6 Hz, H-3), 6.89 (1H, bs, H-7), 6.92 (1H, d, J= 1.7 Hz, H-3'), 6.97 (1H, s, H-4'), 9.09 (1H, bs, NH'), 11.13 (1H, bs, NH). ^{13}C-NMR (DMSO-d_6): δ 96.39 (CH, C-7), 103.90 (CH,C-4'), 105.20 (C, C-7'), 107.75 and 108.01 (CH and CH, C-3 and C-3'), 112.04 (C, C-4), 119.17 and 120.62 (C and C, C-9 and C-9'), 125.07 and 125.65 (C and C, C-2 and C-2'), 131.57 and 132.09 (C and C, C-8 and C-8'), 139.32 (C, C-5), 142.40 and 143.48 (C and C, C-5' and C-6'), 146.17 (C, C-6), 162.30 and 162.53 (C and C, carboxy groups).

2,2'-Dicarboxy-5,5',6,6'-tetrahydroxy-3,4'-biindolyl (7): grey solid; UV: λ_{max} (EtOH) 326 nm (log ε 4.28); HR FAB-MS 385.0676 $(M+H)^+$ (calc. for $C_{18}H_{13}N_2O_8$: 385.0672); ^{1}H-NMR (DMSO-d_6): δ 6.34 (1H, bs, H-3'), 6.38 (1H, s, H-4), 6.80 and 6.81 (1H, s, and 1H, s, H-7 and H-7'), 11.05 and 11.07 (1H, bs and 1H, bs, NH and NH'); ^{13}C-NMR (DMSO-d_6): δ 95.53 and 96.84 (CH and CH, C-7 and C-7'), 104.85 (CH, C-4), 108.11 (CH, C-3'), 113.14 (C, C-4'), 115.36 (C, C-3), 120.64 and 120.87 (C and C, C-9 and C-9'), 123.62 and 124.99 (C and C, C-2 and C-2'), 131.18 and 131.94 (C and C, C-8 and C-8'), 139.35 and 141.42 (C and C, C-5 and C-5'), 145.98 and 146.55 (C and C, C-6 and C-6'), 162.78 and 167.80 (C and C, carboxy groups).

2,2'-Dicarboxy-5,5',6,6'-tetrahydroxy-3,7'-biindolyl (8): grey solid; UV: λ_{max} (EtOH) 320 nm (log ε 4.24); HR FAB-MS 385.0624 $(M+H)^+$ (calc. for $C_{18}H_{13}N_2O_8$: 385.0672); ^{1}H-NMR (DMSO-d_6): δ 6.24 (1H, s H-4), 6.85 (1Hx2, s and s, H-4' and H-7), 6.90 (1H, d, J= 2.0 Hz, H-3'), 8.84 (1H, bs, NH'), 10.84 (1H, bs, NH). ^{13}C-NMR (DMSO-d_6): δ 97.16 (CH, C-7), 102.04 (CH, C-4), 104.33 (CH, C-4'), 107.08 (C, C-7'), 107.65 (CH, C-3'), 116.98 (C, C-3), 119.17 and 119.42 (C and C, C-9 and C-9'), 125.22 and 125.26 (C and C, C-2 and C-2'), 130.28 and 131.61 (C and C, C-8 and C-8'), 141.01 (C, C-5'), 144.57, 144.71, and 144.88 (C, C and C, C-5, C-6 and C-6'), 162.66 and 162.71 (C and C, carboxy groups).

2,2'-Dicarboxy-5,5',6,6'-tetrahydroxy-7,7'-biindolyl (9): grey solid; UV: λ_{max} (EtOH) 319 nm (log ε 4.26); HR FAB-MS 385.0698 $(M+H)^+$ (calc. for $C_{18}H_{13}N_2O_8$: 385.0672) ^{1}H-NMR (DMSO-d_6): δ 6.92 (1Hx2, s, H-4 and H-4'), 6.98 (1Hx2, bs, H-3 and H-3'), 9.18 (1Hx2, bs, NH and NH'). ^{13}C-NMR (DMSO-d_6): δ 103.74 (CH, C-4 and C-4'), 104.46 (C, C-7 and C-7'), 107.85 (CH, C-3 and C-3'), 119.42 (C, C-9 and

C-9'), 126.01 (C, C-2 and C-2'), 131.63 (C, C-8 and C-8'), 142.34 (C, C-5 and C-5'), 144.56 (C, C-6 and C-6'), 162.78 (C, carboxy groups).

REFERENCES AND NOTES

1. G. Prota *Fortschr. Chem Org. Naturst.* **1995**, 64, 94-148.
2. Napolitano, A.; Corradini, M.G.; Prota, G. *Tetrahedron Lett.* **1985**, *26*, 2805-2808.
3. Corradini, M.G.; Napolitano, A.; Prota, G. *Tetrahedron* **1986**, *42*, 2083-2088.
4. d'Ischia, M.; Napolitano, A.; Tsiakas, K.; Prota, G. *Tetrahedron* **1990**, *46*, 5789-5796.
5. Napolitano, A.; Crescenzi, O.; Prota, G. *Tetrahedron*, **1993**, *34*, 885-888.
6. Palumbo, P.; d'Ischia, M.; Crescenzi, O.; Prota G. *Tetrahedron Lett.* **1987**, *28*, 467-470.
7. Palumbo, P.; d'Ischia, M.; Prota, G. *Tetrahedron* **1987**, *43*, 4203-4206.
8. d'Ischia, M.; Napolitano, A.; Prota, G. *Biochim. Biophys. Acta* **1991**, *1073*, 423-430.
9. Cromartie, R.I.T.; Harley-Mason, J. *Biochem. J.* **1957**, *66*, 713-720.
10. Fattorusso, E.; Cimino, G. *Rend. Accad. Sci. Fis. Mat.* **1971**, *38*, 173-182.
11. Ito, S.; Colin Nicol, J. A. *Biochem. J.* **1974**, *143*, 207-217.
12. Structural identification was aided by full assignments of the proton and carbon resonances in the parent 5,6-dihydroxyindole-2-carboxylic acid by 2D NMR techniques. ^{1}H-NMR (DMSO-d_6): δ 6.76 (1H, d, J=0.8 Hz, H-7), 6.81 (1H, dd, J=2.2, 0.8 Hz, H-3), 6.85 (1H, s, H-4), 8.54 (1H, s, OH), 9.05 (1H, s, OH), 11.09 (1H, bs, NH), 12.20 (1H, s, COOH). ^{13}C-NMR (DMSO-d_6): δ 97.29 (CH, C-7), 105.27 (CH, C-4), 107.43 (CH, C-3), 120.22 (C, C-9), 126.13 (C, C-2), 132.97 (C, C-8), 142.32 (C, C-5), 146.46 (C, C-6), 163.03 (C, COOH).
13. Lambert, C.; Chacon, J.N.; Chedekel, M.R.; Land, E.J.; Riley, P.A.; Thompson, A.; Truscott, G. *Biochim. Biophys. Acta* **1989**, *993*, 12-20.
14. Benigni, J.D.; Minnis, R.L. *J. Heterocyclic Chem.* **1965**, *2*, 387-392.

(*Received in UK* 12 *February* 1996; *accepted* 12 *April* 1996)