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Copolymerisation of 5,6-Dihydroxyindole and 5,6-Dihydroxyindole-2-carboxylic Acid in Melanogenesis: Isolation of a Cross-Coupling Product

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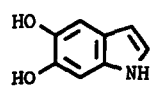
Key Words: 5,6-dihydroxyindoles; oxidative polymerisation; eumelanins; melanogenesis.

Abstract: Under biologically relevant conditions, co-oxidation of 5,6-dihydroxyindole (1) and 5,6-dihydroxyindole-2-carboxylic acid (2) affords, in addition to a complex mixture of homopolymers of the two indoles, a small but significant amount of a cross-coupling product which was isolated as the tetra-O-acetyl derivative and assigned the biindolyl structure 3.

Despite extensive investigations carried out over the years, the structure of eumelanins, the major determinants of skin colour differences in man[1,2], is still open to question. The general view is that the natural pigments are polymers or, more precisely, mixtures of polymers arising mainly from oxidative coupling of 5,6-dihydroxyindole (**1**) and 5,6-dihydroxyindole-2-carboxylic acid (**2**)[2-4]. However, the mode in which the indole units partake in the building-up of the pigment backbone, as well as the nuclear positions involved in the polymerisation have not been definitely assessed.

Recently, we reported the results of model studies showing that under biomimetic conditions homopolymerisation of **1** proceeds preferentially at the 2-, 4- and 7- positions of the indole ring, whereas oxidative coupling of **2** involves invariably at the 4- and 7- positions[5-7].



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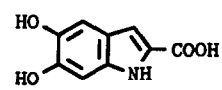

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Using a similar approach, we have now addressed the question whether indoles **1** and **2** can undergo oxidative co-polymerisation, as commonly believed[2-4]. As the oxidizing system for this study, the peroxidase/H_2O_2 couple was preferably used in view of its ability to effect a fast and

complete conversion of 5,6-dihydroxyindoles to melanin pigments[6,8].

Treatment of a mixture of **1** and **2** at 1:1 molar ratio in phosphate buffer, pH 7.0 with 2 molar equivalents of hydrogen peroxide in the presence of horseradish peroxidase (2.1 purpurogallin U/ml) resulted in a rapid consumption of the starting indoles leading in a few minutes to the formation of a dark brown precipitate. HPLC analysis of the oxidation mixture after reduction and acetylation of the ethyl acetate extractable fraction, followed by mild hydrolysis with ethanol/water,[9] revealed a complex but defined pattern of products. Five of the components of the mixture, corresponding to the peaks designated as D, H, I, J, K in the elutogram of fig. 1, were identified as the unchanged acetylated **1** (peak D), the two isomeric dimers (peaks H and I), and the two trimers (peaks J and K), previously obtained in the peroxidase catalysed oxidation of **1**[6].

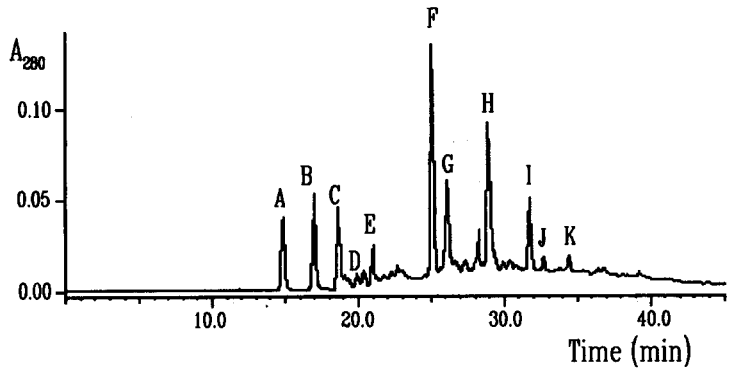
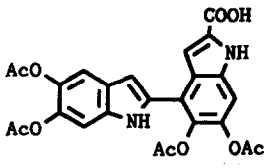


Fig. 1. HPLC elution profile of the products obtained by co-oxidation of 1 and 2 (1:1 molar ratio), followed by acetylation (Spherisorb S5ODS2 column, 250 x 4 mm; gradient: 20 to 70% CH_3CN in 0.1 *M* AcOH, 1 ml/min).

All the other components of the mixture contained an acidic function, seemingly the carboxyl group, as evidenced by separate HPLC analysis of the bicarbonate-extractable fraction. Four of these, compounds A, B, C, and E of the elutogram (fig. 1), proved to be the *O*-acetyl derivative of **2**, and the two related dimers and the trimer previously described as the major products of the enzymic[7,8] or metal-catalysed[7,10] oxidation of **2**, whereas compounds F and G did not correspond to any of the known oligomers of the indole. The most abundant of these, F, was isolated in 5% yield by preparative HPLC purification of the acid fraction of the mixture and formulated as the 5,5',6,6'-tetraacetoxy-2'-carboxy-2,4'-bi-indolyl (**3**)[11] by C-H correlation and selective decoupling experiments.

From consideration of the oxidation chemistry of 5,6-dihydroxyindoles under a variety of conditions[6,7,12,13], including the peroxidase/H_2O_2 induced polymerization, it appears that formation of dimer **3** can



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reasonably be accounted for in terms of ionic type coupling mechanism involving the nucleophilic attack of the 2-position of **1** to the electron deficent C-4 site of the highly reactive indole-5,6-quinone transiently generated by oxidation of **2**. A similar mechanism of incorporation of 2-derived units into the backbone of melanin pigment was invoked by Cromartie and Harley-Mason to account for the observed retention of carboxyl groups in synthetic melanins from tyrosine[14].

Interestingly enough, the yield of formation of dimer **3** was found to increase with respect to that of the oligomers of **2** (HPLC evidence) with increasing the indole **1**/indole **2** molar ratio used in the cooxidation experiments (e.g. 10:1), as would be expected under conditions in which the quinone of **2** is efficiently trapped by the excess nucleophilic species, i.e. **1**, and thus subtracted to the homopolymerisation reaction.

To our knowledge, the isolation of the cross-coupling product **3** reported in this study represents the first evidence that copolymerisation of indoles **1** and **2** can occur under biologically relevant conditions. If the same reactivity of the indole precursors is expressed under the conditions of eumelanogenesis *in vivo*, this would lead to a new concept of natural eumelanins as complex mixtures consisting of homopolymers of **1** and **2** along with copolymers of the two indoles, presumably at different levels of oxidation and polymerisation.

The observed dependence of the distribution of the products of the cooxidation reaction on the molar ratio of the starting indoles would also indicate that the extent to which the copolymer fraction contributes to the whole pigment polymer is dictated by the relative yield of formation of **1** and **2** in the intramolecular rearrangement of the common biosynthetic precursor, dopachrome. In this connection it is noteworthy that the course of the rearrangement reaction was shown to be under the regulatory control of a specific enzyme, dopachrome tautomerase[15], as well as of certain metal ions[16], such as Cu^{2+}, Zn^{2+}, commonly found in melanin containing tissues[17].

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