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Sulphydryl compounds in melanogenesis. Part II. Reactions of cysteine and glutathione with dopachrome.

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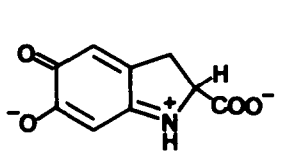
Abstract. Under biomimetic conditions dopachrome (1), a key intermediate in the biosynthesis of melanins, is shown to react with glutathione to give a colourless adduct identified as 4-S-glutathionyl-5,6-dihydroxyindole (3). In the case of cysteine, the reaction leads to a non aminoacidic condensation product (λ max 422 nm) which was too unstable to be characterized. The analogous adduct derived by reaction of dopachrome methyl ester (9a) with cysteine ethyl ester (10a) could be isolated and formulated as 11a, containing the new 1,2-dihydro-3H,8H-pyrrolo [2,3-h] [1,4] benzothiazine ring system. Likewise, 2-methyldopachrome methyl ester (9b) reacts with 10a and penicillamine methyl ester (10b) to give the corresponding adducts 11b or 11c, respectively.

Since the pioneering work of Raper in the twenties, it has been generally agreed that melanins, the characteristic pigments of mammalian skin and hair, are formed by polymerization of various indolic precursors, such as dopachrome (1) and 5,6-indolequinone, derived biogenetically from the tyrosinase-catalyzed oxidation of tyrosine[1]. However, all melanins so far isolated contain a certain amount of sulphur[2] which, as suggested, could probably arise from the trapping of highly reactive melanogenic quinones by sulphydryl compounds commonly found in biological systems[3]. In the preceeding paper[4] we have demonstrated that under biomimetic conditions cysteine and glutathione efficiently react with enzymically or chemically generated 5,6-indolequinone to give 1:1 adducts. As an extension of this study, we report now the reactions of the same sulphydryl compounds with 1, which is the first UV detectable intermediate in the tyrosinase-catalyzed oxidation of tyrosine or dopa[5]. Noteworthy, 1 is a highly unstable aminochrome which, at physiological pH values, undergoes rearrangement with concomitant decarboxylation to give 5,6-dihydroxyindole (2)[6].

In their spectroscopic survey on the reactions of sulphydryl compounds with melanogenic quinones, Bouchilloux and Kodja[7] reported evidence that in the presence of an excess of glutathione, 1 is partially converted into an aminoacidic, o-diphenolic product chromatographically identical with the product formed by reaction of the same thiol with



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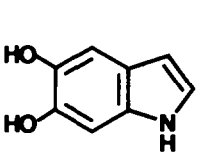

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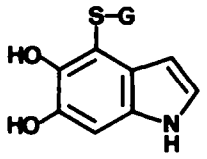

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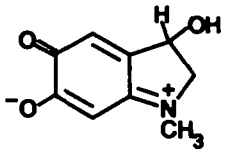

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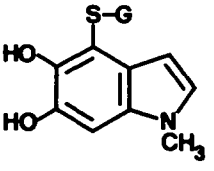

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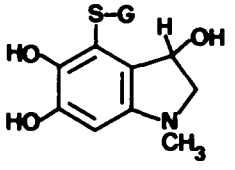

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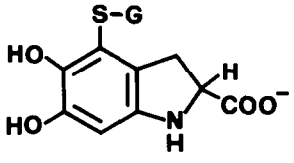

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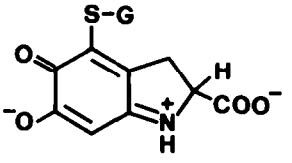

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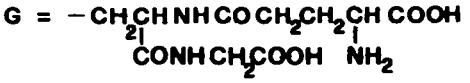
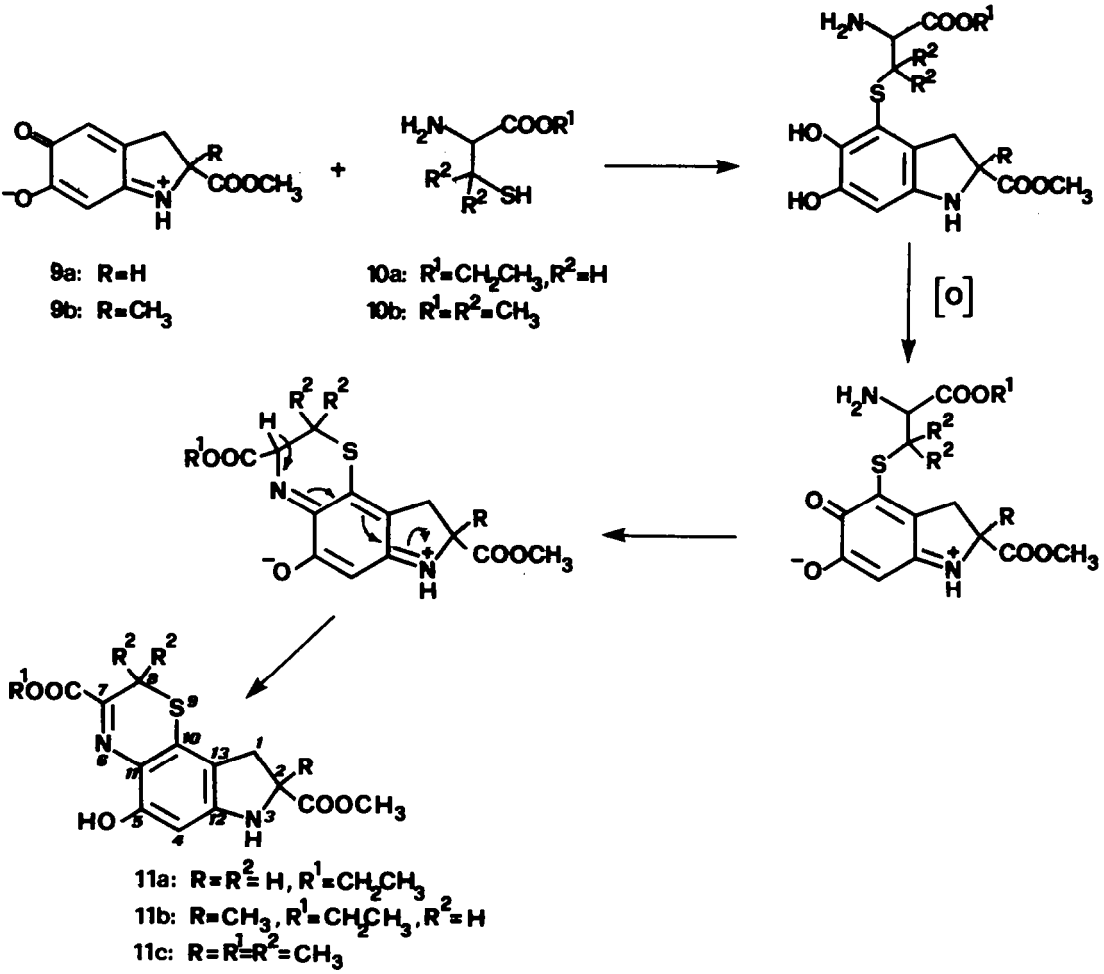


enzymically generated 5,6-indolequinone. On this basis, these authors concluded that, in the presence of glutathione, 1 undergoes rearrangement to 2, which is capable of reacting with the thiol in an oxidizing medium.

When a solution of 1 was allowed to react in phosphate buffer at pH 6.8 with an excess of glutathione, HPLC analysis revealed the formation of 2 (70% yield) along with the reported product (λ max 295 and 307 nm, 15% yield). This proved to be identical with 4-S-glutathionyl-5,6-dihydroxyindole (3), previously obtained[4] by oxidative coupling of 2 with the thiol. In control experiments, it was found that formation of 3 is independent upon the presence of oxygen and that no reaction between 2 and glutathione occurs over a sufficiently prolonged experimental time. This would suggest that the formation of 3 results from a direct addition of glutathione to 1 rather than from a secondary reaction following the rearrangement of 1 to 2 in the presence of glutathione. In this connection, it is noteworthy that a related aminochrome, adrenochrome (4), is reported to react with glutathione to give, inter alia, 4-S-glutathionyl-5,6-dihydroxy-1-methylindole (5), presumably arising by dehydration of an unstable indoline intermediate (6)[8]. In the case of dopachrome, the initial indoline adduct 7 is probably oxidized to the aminochrome 8,[9] which is then converted into 3 by a decarboxylative rearrangement reaction similar to that observed in the case of 1.

A quite different behaviour was observed when 1 was allowed to react with cysteine under the same conditions. After a few minutes the originally red-orange solution turned to yellowish brown and analysis of the reaction mixture revealed the presence of a major non-aminoacidic product with an absorption maximum at 422 nm. A number of attempts to isolate this product were thwarted by its marked instability under the usual chromatographic conditions. However, the corresponding product arising from the reaction of dopachrome methyl ester (9a) with cysteine ethyl ester (10a) could be isolated and identified as 11a, containing the new 1,2-dihydro--3H,8H-pyrrolo [2,3-h][1,4]benzothiazine skeleton.

The UV spectrum of 11a displays an absorption maximum at 435 nm, consistent with the presence of a 2H-1,4-benzothiazine chromophore.[10,11] The ^{1}H-NMR spectrum provides evidence for a highly shielded aromatic proton (singlet at δ 6.09), a $-CH_2-CH<$ grouping (ABX pattern of resonances at δ 3.12, 3.20 and 4.47) and a cyclic methylene group (pair of doublets at δ 3.53 and 3.74). Additional support to structure 11a is provided by the ^{13}C-NMR spectrum exhibiting, besides the signals of the carbomethoxyl and carboethoxylfunctions, six singlets and a doublet in the sp^2 region, two triplets accounting for the C-8 and C-1 methylene groups and a doublet for the C-2.



Scheme

Mechanistically, the formation of 11a can be envisaged as involving the addition of the SH group of 10a to the electrophilic 4-position of the aminochrome to give an indoline intermediate which subsequently undergoes oxidation, ring closure and rearrangement to the 1,2-dihydro-3H,8H-pyrrolo [2,3-h][1,4]-benzothiazine ring system,as depicted in the scheme.An analogous sequence of reactions has been suggested to account for the formation of 2H-1,4-benzothiazines by condensation of β-aminothiols with simple o- and p-benzoquinones.[12,13] Noteworthy, compound 11a in solution rapidly decomposes, giving rise to complex mixtures of red orange oligomeric materials. Such a behaviour is conceivably due to the presence in 11a of two highly reactive ring systems, namely the easily oxidizable 2-carboxyindoline[14] and the 2H-1,4-benzothiazine, which is known to undergo a facile oxidative coupling at the 2-position.[15] If this is the case, one would expect that substitution at the critical 2- and 8-positions on 11a could reduce the susceptibility of the heterocyclic system to autoxidation. Consistent with this view, compounds 11b and 11c, obtained by reaction of 2-methyldopachrome methyl ester (9b) with 10a and penicillamine methyl ester (10b), respectively, proved to be much more stable than the non-methylated derivative 11a under a variety of conditions.

EXPERIMENTAL

UV spectra were measured with a Perkin Elmer Mod. 550 S spectrophotometer. Optical rotations were determined with a Perkin Elmer Mod. 141 polarimeter. ^{1}H-NMR (200 MHz) and ^{13}C-NMR (50 MHz) were recorded on a Varian XL 200 spectrometer (δ values are referred to TMS as an internal standard). Electron impact mass spectra were determined with a Kratos MS-50 mass spectrometer.Besides the molecular ions, the most abundant ions in the mass spectra (above m/e 100) are given with their relative intensities. HPLC analyses were carried out with a Waters model 6000A instrument, using a 4mm x 25 cm RP-18 Lichrosorb column (Merck). The mobile phase was aqueous 0.2 M $Na_2B_4O_7$/methanol (80:20 v/v) adjusted to pH 2.5 with HCl and the flow rate was maintained at 1 ml/min. Detection was carried out with a UV spectrophotometer Waters model 480 (λ =300 nm). Sephadex LH-20 and polyamide used for column chromatography were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), and from Macherey, Nagel and Co., respectively. DL-dopa methyl ester, 2-methyl-L-dopa methyl ester and DL-penicillamine methyl ester were obtained by HCl-methanol esterification of commercially available products. 5,6-Dihydroxyindole was prepared as previously described.[16]

Reaction of dopachrome with glutathione.

Dopachrome was prepared as previously reported[5] by oxidation of DL-Dopa (2.5 mM, 5 ml) with excess silver oxide in 0.025M sodium phosphate buffer, pH 6.8. After filtration the red dopachrome solution (2 mM, 80% yield as determined spectrophotometrically) was freed from traces of silver by batchwise treatment with Chelex 100 resin (Na+ form) and then added to a solution of glutathione (50 mM) in the same buffer (1 ml). The course of the reaction was monitored by HPLC, periodically injecting 5 μl aliquots of the mixture. Two main products were observed and identified as 2 and 3 by analysis of their UV spectra and co-injection with authentic samples. After all dopachrome was decomposed (about 4h), the yields of compounds 2 and 3 were approximately 70% and 15%, as determined by comparison of peak areas with external calibration curves. When anaerobic conditions were required, the solution of dopachrome was placed in a rubber capped quartz cuvette and purged with a nitrogen stream for two minutes. Glutathione was then added via syringe and the purging was continued for further ten minutes. The yields of 2 and 3 were about 75% and 10%, as determined by direct HPLC analysis.

Reaction of dopachrome methyl ester (9a) with cysteine ethyl ester (10a): isolation of 2-carbomethoxy-5-hydroxy-7-carboethoxy-1,2-dihydro-3H,8H-pyrrolo [2,3-h] [1,4] benzothiazine (11a).

Dopachrome methyl ester (9a) was prepared by dropwise addition of a solution of potassium ferricyanide (2.630 g, 8 mmol) in 0.5 M phosphate buffer pH 6.5 (25 ml) to a stirred solution of DL-dopa methyl ester hydrochloride (0.494 g, 2 mmol) in water (25 ml). After 4 min the resulting bright red solution was poured under stirring into a solution of L-cysteine ethyl ester hydrochloride (0.744 g, 4 mmol) in water (10 ml). Within 30 min a yellow-orange precipitate formed, which was collected by centrifugation, washed with water and repeatedly extracted with ethyl acetate. The combined organic layers were evaporated to dryness at 30°C under reduced pressure and the residue, taken up in ethyl acetate, was chromatographed on a 2 x 45 cm Sephadex LH-20 column, using ethyl acetate as the eluent, to give 11a (0.130 g, 20%) as a yellow oil, λmax(MeOH) 281, 434 nm; EIMS m/e: 336 (M+,64), 277 (51), 263 (23), 231 (33), 203 (100); found 336.0774, $C_{15}H_{16}N_2O_5S$ requires 336.0780; ^{1}H-NMR ($CDCl_3$) δ (ppm) : 1.35 (3H, t, J= 7.0 Hz, $-CH_3$), 3.12 and 3.20 (1H, dd, J=16.0, 6.0 Hz and 1H, dd, J = 16.0, 10.0 Hz, $-CH_2-$), 3.53 and 3.74 (1H,d,J= 15 Hz and 1H,d, J=15Hz, $-SCH_2-$), 3.79 (3H,s, $-OCH_3$), 4.33 (2H, q, J= 7.0 Hz, $-OCH_2-$), 4.47(1H, dd, J= 10.0, 6.0 Hz, -CH-),4.87 (1H, bs,-OH), 6.09 (1H, s,H-4); ^{13}C-NMR ($CDCl_3$) δ (ppm) 14.24 (q, $-OCH_2\underline{C}H_3$), 21.94 (t, C-8), 31.59 (t, C-1), 52.67 (q, $-OCH_3$), 59.98 (d,C-2), 61.09 (t, $-OCH_2-$), 92.90 (d,C-4), 114.55 (s, C-13), 121.43 (s, C-11), 122.07 (s, C-10), 136.59 (s, C-7), 153.36 (s, C-12), 156.28 (s, C-5), 163.74 (s, $-\underline{C}OOCH_2CH_3$), 173.70 (s,$-\underline{C}OOCH_3$).

Reaction of 2-methyldopachrome methyl ester (9b) with cysteine ethyl ester (10a): isolation of 2-carbomethoxy-2-methyl-5-hydroxy-7-carboethoxy-1,2-dihydro-3H,8H-pyrrolo [2,3-h] [1,4] benzothiazine (11b).

A solution of 2-methyldopachrome methyl ester (9b), prepared as above by potassium ferricyanide oxidation of 2-methyl-L-dopa methyl ester hydrochloride (0.522 g, 2 mmol),was added under stirring to a solution of L-cysteine ethyl ester hydrochloride (0.744 g, 4 mmol) in water (10 ml). After 30 min the yellow precipitate formed, was collected by centrifugation , washed with water, and repeatedly extracted with ethyl acetate. The combined organic layers were evaporated to dryness at 30°C under reduced pressure and the residue, taken up in ethyl acetate, was chromatographed on a 1.5x25 cm polyamide column using ethyl acetate as the eluent, to give pure 11b (0.175 g, 25% yield) as a yellow oil,λmax(MeOH) 288, 435 nm; $[\alpha]_D^{25}$= +393° (c= 0.9, $CHCl_3$); EIMS m/e 350 (M+,59), 291 (100), 245 (47), 217 (62); found 350.0934, $C_{16}H_{18}N_2O_5S$ requires 350.0936;^{1}H-NMR ($CDCl_3$) δ(ppm) 1.38 (3H,t,J=7.0 Hz, $-OCH_2\underline{C}H_3$), 1.56 (1H,s, $-CH_3$), 2.79 and 3.40(1H, d, J=16.5 Hz and 1H,d,J=16.5 Hz, $-CH_2-$), 3.69 and 3.79 (1H, d,J=15 Hz and 1H,d,J= 15 Hz, $-SCH_2-$), 3.75 (3H, s, $-OCH_3$), 4.36 (2H,q, J=7.0 Hz, $-OCH_2-$), 5.00 (1H,bs,OH),6.05 (1H,s, H-4);^{13}C-NMR ($CDCl_3$) δ(ppm) 14.21 (q,$-OCH_2\underline{C}H_3$), 21.96 (t, C-8), 26.50 (q, $-CH_3$), 38.69 (t, C-1), 52.84 (q, $-OCH_3$), 62.18 (t, $-OCH_2-$), 67.75 (s, C-2), 92.67 (d, C-4), 114.62 (s, C-13), 121.78 (s, C-11), 122.46 (s,C-10), 137.01 (s, C-7), 152.67 (s, C-12), 156.20 (s, C-5), 164.15 (s,$-\underline{C}OOCH_2CH_3$), 175.85 (s,$-\underline{C}OOCH_3$).

Reaction of 2-methyldopachrome methyl ester (9b) with penicillamine methyl ester (10b): isolation of 2,7-dicarbomethoxy-2,8,8-trimethyl-5-hydroxy-1,2-dihydro-3H,8H-pyrrolo [2,3-h] [1,4]benzothiazine (11c).

Similarly, reaction of 2-methyldopachrome methyl ester with penicillamine methyl ester hydrochloride (0.792 g,4 mmol) gave, after work up as above, compound 11c (109 mg, 15% yield) as a yellow oil,λ max(MeOH) 288, 406 nm; $[\alpha]_D^{25}$= +93° (c=0.6, $CHCl_3$); EIMS m/e 364 (M+,32), 305 (100), 245(54); found 364.1087, $C_{17}H_{20}N_2O_5S$ requires 364.1092;^{1}H-NMR ($CDCl_3$) δ (ppm) 1.55 (3H,s, $-CH_3$), 1.57 (3H,s,$-CH_3$), 1.59 (3H,s,$-CH_3$), 2.76 and 3.37 (1H,d,J=15.2 Hz and 1H,d,J= 15.2 Hz,$-CH_2-$), 3.73 (3H,s,$-OCH_3$),3.84 (3H,s,$-OCH_3$), 4.84 (1H,bs,-OH), 6.08 (1H,s, H-4);^{13}C-NMR ($CDCl_3$) δ(ppm) 26.14 (q,$-CH_3$), 26.25 (q, $-CH_3$), 26.60 (q,$-CH_3$), 38.47 (t,C-1), 40.50 (s, C-8), 52.28 (q,$-OCH_3$), 52.77 (q, $-OCH_3$), 67.56 (s, C-2), 92.38 (d, C-4), 113.83 (s,C-13), 120.44 (s, C-11), 122.54 (s, C-10), 146.67 (s, C-7), 152.21 (s, C-12), 154.95 (s, C-5), 164.37 (s,$-\underline{C}OOCH_3$), 175.94 (s, $-\underline{C}OOCH_3$).

ACKNOWLEDGEMENTS

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