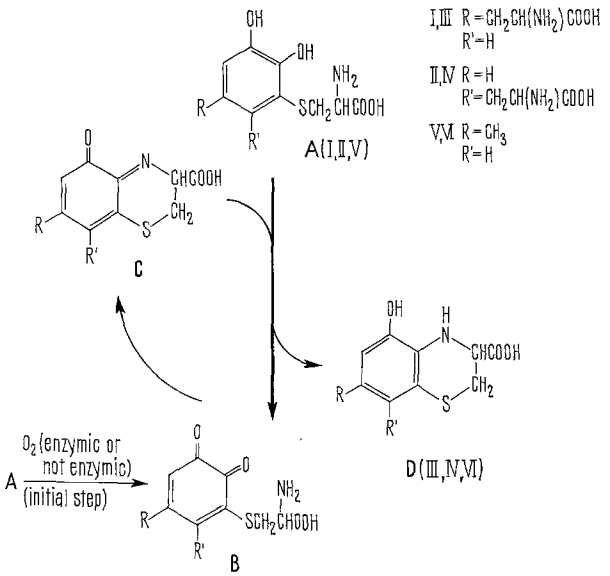
New Intermediates in Phaeomelanogenesis in vitro[1]

It has recently been established that phaeomelanins[2] are formed in vivo by a deviation of the eumelanin pathway involving the 1,6-addition of cysteine to dopaquinone produced by enzymic oxidation of tyrosine[3-5]. Consequently the first step of phaeomelanogenesis leads to the formation of 5-S-cysteinyldopa[6] (I) and 2-S-cysteinyldopa[4] (II), the relative ratio of which has been shown to be approximately 95 to 5.

In this report, in vitro experiments are described which suggest that the next step of phaeomelanin biosynthesis involves the cyclization of the cysteinic residue of I and II to give, as outlined in scheme 1, the dihydrobenzothiazines III and IV. These results were obtained by studying the course of the enzymic oxidation of I and II to synthetic phaeomelanins and the conversion of a model compound[7] (V) into the corresponding dihydrobenzothiazine (VI).



Enzymic oxidation of 5-S-cysteinyldopa (I). A solution of I (100 mg) in 0.3 *M* acetate buffer (50 ml) at pH 6.8 was oxidized[8] in the presence of mushroom polyphenoloxidase (1000 catecholase units) and the course of the oxidation followed spectrophotometrically. Within a few min, the starting product (I, λ_{max} 292, 255 nm) was completely converted into a colourless product, the absorption spectrum of which ($\lambda_{max}^{pH\ 6.8}$ 306 nm; $\lambda_{max}^{N\ HCl}$ 292, 286 nm) was similar to those of some model 5-hydroxy-3,4-dihydro-2H-1,4-benzothiazines, described previously[7,9]. At this stage the oxidation was stopped by addition of $Na_2S_2O_4$ and the resulting solution passed through a column of Amberlite CG-50 (H^+ form). The vacuum-concentrated eluate was then fractionated by chromatography on Sephadex G-25 column (2 × 60 cm) using as eluent 0.1 *N* HCl. The fractions containing the product under investigation (analyzed by UV-spectrometry) were further purified by chromatography on Sephadex G-25 (eluent 0.4 *M* CH_3COOH) to give 35 mg of an amorphous, hygroscopic powder (III), which darkens rapidly on standing. (Found: C, 47.5; H, 5.0; N, 9.1; S, 10.5. $C_{12}H_{14}N_2O_5S$ requires: C, 48.3; H, 4.7; N, 9.4; S, 10.7%.)

The purified product gave a grey-purple colour with ninhydrin and a dark green one with $FeCl_3$. Esterification of III (5 mg) with ethanol, catalyzed by HCl (48 h at room temperature), afforded a diethylester, $C_{16}H_{22}N_2O_5S$ (by high resolution mass spectrometry[10]), which gave a single spot (Rf 0.40, located by UV-light) when analyzed by TLC on silica (F_{254}, Merck) with 5% EtOH-$CHCl_3$. The mass spectrum of this product showed the molecular ion peak at *m/e* 354 and diagnostic fragment ions at *m/e* 252 (base peak, $M^+ - \cdot CH(NH_2)COOC_2H_5$) and *m/e* 179 ($252 - \cdot COOC_2H_5$).

Considering the above data, the structure of β-7-(3-carboxy-5-hydroxy-3,4-dihydro-2H-1,4-benzothiazinyl)-alanine was tentatively assigned to compound III.

Enzymic oxidation of 2-S-cysteinyldopa (II). The spectrophotometric course of the oxidation of II (30 mg) was found to be identical with that observed in the case of 5-S-cysteinyldopa; after about 6 min the oxidation was stopped by addition of $Na_2S_2O_4$ and the resulting solution passed through a column of Amberlite CG-50. The eluate was evaporated to dryness and the residue esterified with ethanol as in the preceding experiment. The crude product was then purified by chromatography on a 1 × 25 cm polyamide (Macherey, Nagel and Co.) column using as eluent 50% C_6H_6-$CHCl_3$ and the fractions containing the product under investigation (λ_{max} 306 nm) were combined and evaporated to dryness. The product (7 mg) was characterized as β-8-(3-carbethoxy-5-hydroxy-3,4-dihydro-2H-1,4-benzothiazinyl)-alanine ethyl ester on the basis of the mass spectrum which showed the molecular ion peak at *m/e* 354 ($C_{16}H_{22}N_2O_5S$ by high resolution mass spectrometry) and fragment ions at *m/e* 252 and 179. When analyzed by TLC on silica in various solvents, the product gave a single spot (located by UV-light) having an Rf value identical with that of the diethyl ester of compound III.

Oxidation of I and II with O_2 in alkaline media. A stream of O_2 was bubbled into a solution of I (or II) in phosphate buffer (pH 8.3). After about 20 min, the initial absorption maxima of I (or II) at 292, 255 nm were replaced by a maximum at 306 nm which was hypsochromically shifted to 292, 286 nm on addition of HCl.

Oxidation of 3-[(5,6-dihydroxy-m-tolyl)thio]-L-alanine (V). To support further the structures assigned (III and IV), a solution of the model compound V (87 mg) in 0.3 *M* acetate buffer (40 ml) was oxidized in the presence of mushroom polyphenoloxidase (600 U). Within a few min the absorption maxima of V at 292, 255 nm were replaced by a maximum at 306 nm and then the oxidation was stopped by addition of $Na_2S_2O_4$. The resulting solution

[1] This investigation was supported by a grant from the Laboratorio di Chimica e Fisica per lo Studio delle Molecole di Interesse Biologico del C.N.R., Napoli.

[2] R. A. NICOLAUS, in *Melanins* (Ed. E. LEDERER; Hermann, Paris 1968), p. 119.

[3] G. MISURACA, R. A. NICOLAUS, G. PROTA and G. GHIARA, Experientia *25*, 920 (1969).

[4] E. FATTORUSSO, L. MINALE, S. DE STEFANO, G. CIMINO and R. A. NICOLAUS, Gazz. chim. ital. *99*, 969 (1969).

[5] G. PROTA, J. invest. Derm. *50*, 82 (1970).

[6] G. PROTA, G. SCHERILLO and R. A. NICOLAUS, Gazz. chim. ital. *98*, 495 (1968).

[7] G. PROTA, G. SCHERILLO, E. NAPOLANO and R. A. NICOLAUS, Gazz. chim. ital. *97*, 1451 (1967).

[8] Preliminary experiments showed that the presence of a catalytic amount of 3,4-dihydroxyphenyl-L-alanine[4] was not required for the formation of the below-mentioned intermediate.

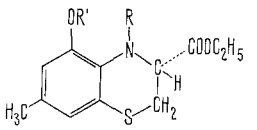
[9] G. PROTA, O. PETRILLO, C. SANTACROCE and D. SICA, J. heterocyclic Chem. *7*, 555 (1970).

[10] Mass spectra and accurate mass measurements were obtained with an A.E.I. MS-902 double focus spectrometer by using the direct inlet technique.

was then acidified to pH 2.5 and extracted with ether. Removal of the solvent gave 76 mg (94% yield) of an amorphous powder (VI) $C_{10}H_{11}NO_3S$ (by high resolution mass spectrometry), $[\alpha]_D$ — 33.6° (0.54% in C_2H_5OH; λ_{max}^{EtOH} 306, 230 nm (log ε, 3.35, 4.31); $\lambda_{max}^{N\,HCl}$ 291, 283, 252, 217 nm (log ε, 3.57, 3.61, 3.94, 4.36).

Similar yields (96%) of compound VI were also obtained when a solution of V (0.87 g) in phosphate buffer at pH 8.2 (80 ml) was oxidized with O_2 for 20 min at room temperature.

Characterization of VI. Crystalline stable derivatives of VI were obtained as follows: a solution of VI (500 mg) in anhydrous ethanol was saturated with HCl and the mixture left at room temperature for 48 h. The crude ethyl ester (450 mg) was then treated (70 h, room temperature) with acetic anhydride (30 ml) and pyridine (20 ml). Removal of the solvents left an oil which was separated by PLC over silica (F_{254}, Merck) with 50% ether-chloroform to give three products (Rf 0.64, 0.54 and 0.30; located by UV-light) which were characterized as VII, VIII and IX, respectively, on the basis of the following evidence.



VII; R=H, R'=COCH$_3$
VIII; R=R'=COCH$_3$
IX; R=COCH$_3$, R'=H

Compound VII (216 mg), analyzed for $C_{14}H_{17}NO_4S$[11] (M^+ 295), formed prisms from ethanol, mp 76–77 °C, $[\alpha]_D$ — 49.2° (0.51% in $CHCl_3$); λ_{max}^{EtOH} 311, 231 nm (log ε, 3.58, 4.36); ν_{max} (CCl_4) 3370 (NH), 1775 ($OCOCH_3$), 1745 ($COOC_2H_5$) cm^{-1}; NMR (CCl_4): δ 1.23 (3H, *t*, J ≃ 7, OCH_2–CH_3), 2.18 (6H, *s*, $ArCH_3$ and $OCOCH_3$), 3.0 (2H, unresolved 8-lines *m*, S–CH_2–CH, AB part of an ABX system), 4.10 (1H, *dd*, S–CH_2–CH, X part of the ABX system), 4.15 (2H, *q*, J ≃ 7, OCH_2–CH_3), 4.55 (1H, *br s*, NH), 6.55 (1H, aromatic proton), 6.58 (1H, aromatic proton).

Compound VIII (222 mg), $C_{16}H_{19}NO_5S$ (M^+ 337), mp 155–157 °C (from ethanol), $[\alpha]_D$ — 86.1° (0.50% in $CHCl_3$); λ_{max}^{EtOH} 261, 232 nm (log ε, 4.04, 4.38); ν_{max} ($CHCl_3$) 1770 ($OCOCH_3$), 1745 ($COOC_2H_5$), 1665 ($NCOCH_3$) cm^{-1}; NMR ($CDCl_3$): δ 1.17 (3H, *t*, J ≃ 7, OCH_2–CH_3), 2.02 (3H, *s*, N–$COCH_3$), 2.29 (6H, *s*, $ArCH_3$ and $OCOCH_3$), 2.97 and 3.46 (2H, two *q*, J_{AB} ≃ 13, J_{AX} ≃ 9, J_{BX} ≃ 8, S–CH_2–CH), 4.10 (2H, *q*, J ≃ 7, OCH_2–CH_3), 5.64 (1H, *dd*, S–CH_2–CH), 6.93 (1H, aromatic proton), 7.09 (1H, aromatic proton).

Compound IX (31 mg), $C_{14}H_{17}NO_4S$ (M^+ 295), mp 120 to 122 °C (from ethanol); λ_{max}^{EtOH} 287 (sh), 262, 228 nm (log ε, 3.73, 3.99, 4.38); ν_{max} ($CHCl_3$) 3330 (OH), 1720 ($COOC_2H_5$), 1665 (N–$COCH_3$) cm^{-1}.

Discussion. The results reported in this study show evidence that, under the conditions of the in vitro phaeomelanogenesis, 5-S-cysteinyldopa (I) as well as 2-S-cysteinyldopa (II) are rapidly converted into the corresponding dihydrobenzothiazines III and IV, which are the first UV-detectable intermediates preceding the appearance of pigments. The assignment of the structures III and IV for these new intermediates is mainly based on their absorption spectra and on the mass spectra of the corresponding diethyl esters, because the instability of the products has precluded chemical characterization. However, indirect evidence for the proposed structures is provided by the study of the model compound (V), the cyclization product (VI) of which has been unequivocally characterized by spectral and chemical data.

Considering that the cyclization of the cysteinic residue of the model compound (V), as well as of the phaeomelanin precursors (I and II), takes place almost quantitatively[12] within a few min, a self-catalyzed reaction involving oxidation and reduction steps is likely for the formation of dihydrobenzothiazines (scheme 1). Presumably, the cyclization reaction is initiated by the formation of a catalytic amount[13] of *o*-quinone (B) which undergoes an intramolecular elimination of water producing as intermediate an *o*-quinonimine derivative (C); this may be reduced to dihydrobenzothiazine by the starting product which in turn is oxidized to the corresponding *o*-quinone and the reaction continues spontaneously.

Further experiments are now required to clarify the pathway by which the dihydrobenzothiazines III and IV, the existence of which has not been described previously, are oxidized in the subsequent steps of the in vitro phaeomelanogenesis.

Riassunto. Mediante esperimenti in vitro, è stato accertato che i primi stadi della biosintesi delle feomelanine conducono alla formazione di derivati diidrobenzotiazinici, ai quali sono state assegnate le strutture III e IV.

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[11] Satisfactory C, H, N and S analyses of VII, VIII and IX were obtained. IR-spectra were recorded on a Perkin-Elmer Infracord (mod. 137 E) and NMR-spectra on a Varian A-60 apparatus (internal reference TMS); *s* denotes a singlet, *d* a doublet, *t* a triplet, *q* a quartet, *m* a multiplet, *dd* a doublet of doublets, *br* broad.

[12] The quantitative conversion of I (or II) into III (or IV) can be observed spectrophotometrically, but the unfavourable properties of the product precludes its isolation in very high yields.

[13] As expected, manometric experiments showed that only a catalytic amount of oxygen is required during the cyclization reaction.

The Synthesis of Monofluoroacetic Acid by a Tissue Culture of *Acacia georginae*

Monofluoroacetic acid is a highly toxic compound to mammals and has been detected in several plant species from Australia, South Africa, and Brazil[1]. The assumption that the synthesis of this compound may be a response to high concentrations of available inorganic fluoride in the soil or water appears to be supported by the observations of Cheng et al.[2] and Lovelace et al.[2] who have reported the presence of fluoroacetate in the foliage of soybeans and common forage plants exposed to gaseous inorganic fluoride.

Peters et al.[3] and Preuss[3] have described the in vivo synthesis of fluoroacetate by *Acacia georginae* grown