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# A New Amino Acid, 3-(2,5-*SS*-Dicysteinyl-3,4-dihydroxyphenyl)alanine, from the Tapetum lucidum of the Gar (Lepisosteidae) and its Enzymic Synthesis

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The tapetum lucidum of the alligator gar *Lepisosteus* was shown by t.l.c. to contain a new phenolic amino acid, which is apparently a major constituent of the reflecting material. It was isolated in a yield of 0.5 mg/eye and its physical and chemical characteristics, especially reductive hydrolysis with hydriodic acid giving dopa (3,4-dihydroxyphenylalanine) and cysteine, suggested that it might be *SS*-dicysteinyldopa. Tyrosinase oxidation of L-dopa in the presence of an excess of L-cysteine yielded, in addition to known 5- and 2-*S*-cysteinyldopa, the same amino acid as that isolated from the eye of the gar, thus confirming the gross structure. The position of the two cysteine residues was established by the fact that tyrosinase oxidation of catechol and cysteine gave 3-*S*-cysteinylcatechol and 3,6-*SS*-dicysteinylcatechol. The natural amino acid is therefore formulated as 3-(2,5-*SS*-dicysteinyl-3,4-dihydroxyphenyl)alanine (2,5-*SS*-dicysteinyldopa), which may be formed by two consecutive additions of cysteine, first to dopaquinone and then to 5-*S*-cysteinyldopaquinone. The enzymic synthesis of 2,5-*SS*-dicysteinyldopa *in vitro* suggests that it may also be involved in the biosynthesis of phaeomelanin.

The tapetum lucidum of the eye is a specialized layer, which lies behind the light-sensitive cells of the retina (Walls, 1942; Pirie, 1966). The chemistry of the tapetum lucidum is quite diverse and among fishes we have found, in addition to guanine, which has long been known (Walls, 1942), several distinctive reflecting materials: a trigylceride (Arnott *et al.*, 1972); a pteridine (Zyznar & Nicol, 1973); and oligomers of 5,6-dihydroxyindole-2-carboxylic acid associated with *S*-adenosyl-3-methylthiopropylamine (Ito & Nicol, 1974, 1975*a*).

Nicol & Arnott (1973) have reported that eyes of gars (*Lepisosteus*) possess a yellow tapetum lucidum, which is located in processes of the pigment epithelium. A yellow reflecting material is enclosed in tapetal spheres. In the present paper we describe t.l.c. analysis of the tapetum lucidum, the isolation of a major constituent of the reflecting material (designated as amino acid A) and the elucidation of its structure. A preliminary report of this study has appeared (Ito & Nicol, 1975*b*).

## Experimental

### *Materials*

*Animals.* Alligator gars *Lepisosteus spatula* (Lacépède) were caught in traps and gill nets in rivers of south Texas. Whole eyes for the preparative-scale extraction were stored at −20°C until required.

*Chemicals.* L-Cysteine, L-dopa (L-3,4-dihydroxyphenylalanine) and mushroom tyrosinase (type III, 2750 Sigma units/mg; EC 1.14.18.1) were from Sigma Chemical Co., St. Louis, MO, U.S.A.

### *Chromatography*

Precoated plates (microcrystalline cellulose and silica gel) for t.l.c. were from E. Merck, Darmstadt, West Germany. The solvent systems used were (by vol.): (1) propan-1-ol/1 M-HCl (2:1); (2) propan-1-ol/1 M-HCl (3:2); (3) propan-1-ol/1 M-HCl (1:1); (4) butan-1-ol/acetic acid/water (12:3:5); (5) butan-1-ol/acetic acid/water/6M-HCl (20:30:50:2; Prota *et al.*, 1968); (6) 90% formic acid/methanol/conc. HCl (15:3:1, Butenandt *et al.*, 1958); (7) propan-1-ol/conc. aq. $NH_3$ (sp. gravity 0.88)/water (6:3:1). Compounds were detected with either a u.v. lamp (254nm), ninhydrin reagent or 3% (w/w) $FeCl_3$ solution for *o*-diphenols.

### *Instrumental analysis*

All melting points are uncorrected. Elementary analyses were performed by Alfred Bernhard, Microanalytical Laboratory, Mülheim, West Germany. Optical rotations were measured on a Perkin–Elmer 141 polarimeter. U.v.-absorption spectra were taken on a Beckman model 24 spectrophotometer, and i.r.-absorption spectra on a Perkin–

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Elmer grating i.r. spectrophotometer model 237B. N.m.r. (nuclear-magnetic-resonance) spectra were recorded on a Varian HA-100 spectrometer. The chemical shifts are given in p.p.m. relative to tetramethylsilane in [$^2$H]chloroform. When the spectrum was taken in 1M-$^2$HCl, 2-methylpropan-2-ol was used as an internal standard [$\delta$1.28 relative to 3-(trimethylsilyl)propionic acid (sodium salt) relative to which all the chemical shifts are expressed]. The coupling constants are given in Hz.

*T.l.c. analysis of the tapetum lucidum*

One dark-adapted eye of an alligator gar (total body length 78cm) was opened and the retina removed, thereby exposing the pigment epithelium. The latter was gently brushed in 0.25M-sucrose and the resulting suspension (4ml) was homogenized in a TenBroeck tissue grinder (Kimble no. 43930, Toledo, OH, U.S.A.). The milky light-tan homogenate was centrifuged at 15000*g* for 10min at 3°C. The pellet was washed with 1ml of water by centrifugation at 15000*g* for 10min at 3°C and extracted with 2×4ml of 1M-formic acid. The formic acid extract was concentrated to near dryness under reduced pressure at 40°C, transferred to a small test tube and evaporated to dryness in a vacuum desiccator. The residue was dissolved in 0.5ml of propan-1-ol/1M-HCl (3:2, v/v); t.l.c. of this tapetum extract (20$\mu$l) is shown in Fig. 1.

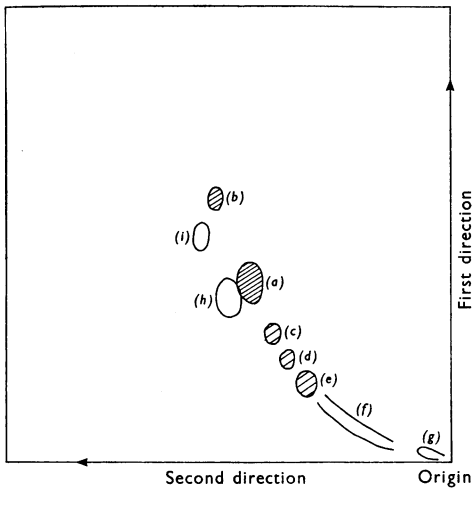



Fig. 1. *Two-dimensional t.l.c. of tapetum extract*
Tapetum extract of the gar was chromatographed on a cellulose plate (20cm×20cm). It was developed first in propan-1-ol/1M-HCl (1:1, v/v) for 11h and secondly in butan-1-ol/acetic acid/water/6M-HCl (20:30:50:2, by vol.) for 5.5h; the solvent fronts reached the edge in both directions. The chromatogram was first examined under a u.v. lamp (354nm) and then sprayed with either ninhydrin or $FeCl_3$ solution. All the spots that were detected under the u.v. lamp are shown. The spots that gave violet colours with ninhydrin and green ones with $FeCl_3$ are indicated by cross-hatching, which also indicates the intensities of the colours developed by the spray reagents. Spot (*f*) weakly ninhydrin-positive; spot (*g*) weak brown (visible); spot (*h*) intense blue fluorescence (u.v.).

The black tissue exposed after brushing (see above) was also analysed. It was homogenized with 1ml of 1M-HCl and centrifuged to give choroid extract. Samples of tapetum extract (20$\mu$l) and choroid extract (10$\mu$l) were analysed by t.l.c. in solvents (2), (5) and (7); guanine, hypoxanthine, xanthine and adenine were also chromatographed for identification.

*Isolation of amino acid A from whole eyes*

To characterize what appeared to be the major component of the tapetum extract [amino acid A, spot (*a*) in Fig. 1] a large-scale separation was made.

For this purpose 50 whole eyes (wet wt. 136g) of alligator gars were homogenized in a Vir-Tis 23 homogenizer (Vir-Tis Co., Gardiner, NY, U.S.A.) and extracted with 6vol. (v/v) of 1M-HCl at room temperature (about 23°C) for 2h. The suspension was centrifuged at 10000*g* for 10min and the cloudy supernatant filtered through sintered glass. The yellow filtrate was applied to a column (2.2cm×8.0cm) of Dowex-50W (X4, $H^+$ form, 200–400 mesh). The column was washed with 1500ml of 1M-HCl and the fraction containing amino acid A was eluted with 1250ml of 6M-HCl, which was then evaporated to dryness under reduced pressure at 50°C. The residue was taken up in methanol/1M-HCl (19:1, v/v) and chromatographed on a column (4.1cm×50cm) of Sephadex LH-20, with methanol/1M-HCl (19:1, v/v) as eluent (Fig. 2). Fractions (10ml) were monitored by their $A_{305}$. Fractions 36–41 containing the amino acid A were combined and evaporated to dryness under reduced pressure. The residue was again chromatographed on the Sephadex LH-20 column under identical conditions. Fractions 36–42 were evaporated and applied to Whatman 3MM paper, which was developed in solvent (2). A major u.v.-quenching band, which contained the amino acid A, was eluted with 0.1M-HCl and evaporated to dryness under reduced pressure. The crude amino acid hydrochloride was then converted into the formate salt by passage through a column (0.5cm×6cm) of Dowex-2 (X8, formate form, 100–200 mesh) and the formate salt was further purified by chromatography on a column (2.2cm×64cm) of Sephadex G-25 with 0.5M-formic acid as eluent. Fractions (5ml) were monitored by $A_{305}$. Fractions 33–43 were combined, evaporated to a small volume under reduced pressure and then freeze-dried. Drying at 40°C under reduced pressure (1mm Hg) yielded 25.6mg of 2,5-*SS-dicysteinyldopa formate* as

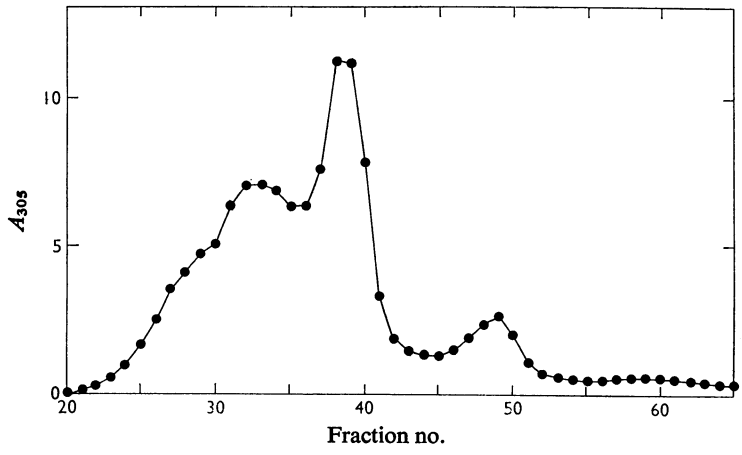



Fig. 2. *Elution pattern from a Sephadex LH-20 column of extract of whole eyes*
Fractions 36–41 contained the compound from spot (*a*); 25–30, the compounds from spots (*f*) and (*g*); 31–35, the compounds from spots (*c*), (*d*) and (*e*); 45–52, the compound from spot (*b*). See the Experimental section for further details.

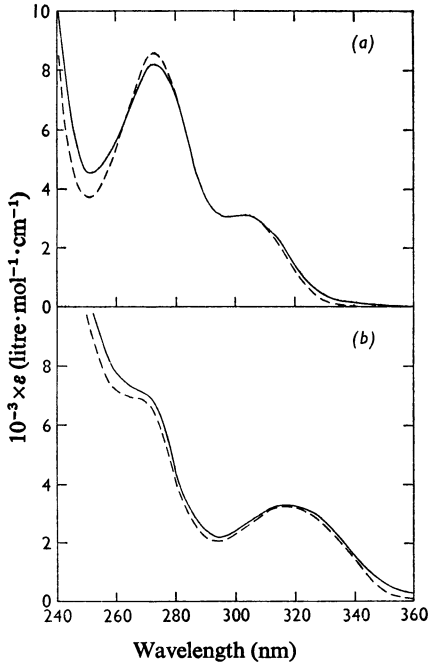



Fig. 3. *U.v.-absorption spectra of natural and synthetic amino acid A* (2,5-*SS-dicysteinyldopa*)
——, 2,5-*SS*-Dicysteinyldopa isolated from eyes of the gar: (*a*) $\lambda_{max.}$ 217, 273 and 303nm ($\varepsilon$ 23400, 8200 and 3100 litre·mol$^{-1}$·cm$^{-1}$); (*b*) $\lambda_{max.}$ 223, 267 (shoulder) and 316nm ($\varepsilon$ 22800, 7200 and 3300 litre·mol$^{-1}$·cm$^{-1}$). ----, 2,5-*SS*-Dicysteinyldopa prepared by the enzymic synthesis: (*a*) $\lambda_{max.}$ 217, 273 and 302nm ($\varepsilon$ 22700, 8600 and 3100 litre·mol$^{-1}$·cm$^{-1}$); (*b*) $\lambda_{max.}$ 223, 267 (shoulder) and 316nm ($\varepsilon$ 22100, 6900 and 3200 litre·mol$^{-1}$·cm$^{-1}$). The spectra were taken in (*a*) 0.1M-HCl and (*b*) 0.05M-sodium phosphate buffer, pH6.8, containing 0.01M-mercaptoethanol. The largest absorption maxima below 230nm were omitted from the spectra for clarification.

an amorphous colourless powder, $[\alpha]_D^{20} = +113°$ (*c* 0.36 in 1M-HCl) (Found: C, 39.7; H, 5.3; N, 8.8; S, 13.3; ash, 0.5. $C_{15}H_{21}N_3O_8S_2,HCO_2H$ requires C, 39.9; H, 4.8; N, 8.7; S, 13.3%). Its $R_F$ values in solvents (2), (5) and (6) were 0.16, 0.48 and 0.54 respectively (those of cystine were 0.28, 0.61 and 0.64 respectively). The u.v.-absorption spectra are shown in Fig.3 and the n.m.r. spectrum (part) is shown in Fig. 4.

*Reductive hydrolysis with hydriodic acid of amino acid A* (2,5-*SS-dicysteinyldopa*)

A solution containing 2.6mg of the amino acid A in 1ml of freshly distilled hydriodic acid containing 10mg of red phosphorus was heated at 110°C in an evacuated sealed tube for 24h. The hydriodic acid was evaporated under reduced pressure at 60°C and 1–2ml of water was added twice and evaporated. The residue was taken up in 0.5–1ml of 0.1M-HCl, filtered and the filtrate evaporated in a vacuum desiccator over KOH pellets. Small portions of the products were analysed by t.l.c. in solvents (1) and (4). Cysteine, dopa and 5-*S*-cysteinyldopa (Prota *et al.*, 1968) were identified. To confirm the identification of dopa and 5-*S*-cysteinyldopa and estimate their

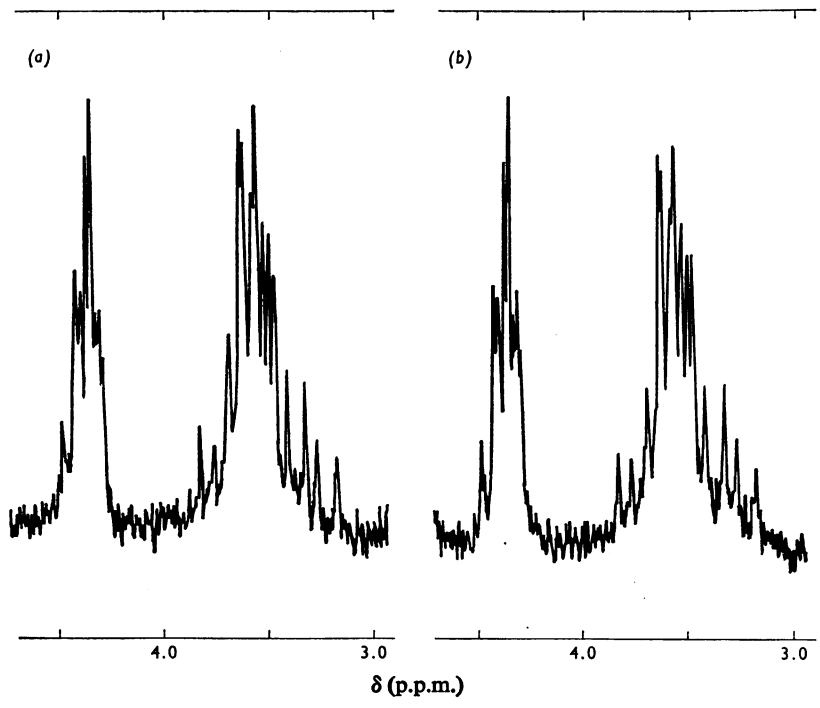



Fig. 4. *N.m.r. spectra of natural and synthetic amino acid A* (2,5-*SS*-*dicysteinyldopa*)
(*a*) 2,5-*SS*-Dicysteinyldopa isolated from eyes of the gar; (*b*) 2,5-*SS*-dicysteinyldopa prepared by the enzymic synthesis. Both spectra were taken in 1 M-$^2$HCl and only $\delta$ 3.0–4.5 regions are shown.

yields the products were chromatographed on Whatman 3MM paper in solvent (1). Two bands were detected under a u.v. lamp and each was eluted with 10ml of 0.1M-HCl. The yields were spectrophotometrically determined.

*Enzymic synthesis of 5-S-cysteinyldopa, 2-S-cysteinyldopa and 2,5-SS-dicysteinyldopa*

Mushroom tyrosinase (33mg) was added to a solution containing 197mg (1mmol) of L-dopa and 363mg (3mmol) of L-cysteine in 120ml of 0.05M-sodium phosphate buffer, pH6.8, and the solution was stirred at 22°C ($O_2$ was not bubbled into the solution). The reaction was followed by u.v. spectrophotometry (Fig. 5). The reaction was allowed to proceed for 10h and stopped by the addition of 2ml of 6M-HCl. The insoluble material formed was filtered off and the filtrate was passed through a column (2.2cm×5.7cm) of Dowex-50W (X4, $H^+$ form, 200–400 mesh). The column was eluted with 600ml of 1M-HCl; the first 400ml were discarded and the 400–600ml fraction was saved for the isolation of 2-*S*-cysteinyldopa (see below). 5-*S*-Cysteinyldopa and 2,5-*SS*-dicysteinyldopa were then eluted with 600ml of 6M-HCl and evaporated to dryness under reduced pressure at 50°C. The residue was chromatographed on the Sephadex LH-20 column (as described above). Fractions 35–40 and 44–53 contained 2,5-*SS*-dicysteinyldopa and 5-*S*-dicysteinyldopa respectively. The three fractions containing 2-*S*-cysteinyldopa, 5-*S*-cysteinyldopa and 2,5-*SS*-dicysteinyldopa were separately chromatograhed on Whatman 3MM paper in solvent (2). Extraction in 0.1M-HCl of the appropriate u.v.-quenching bands of material and evaporation of the subsequent extracts yielded 269mg (68% yield) of 5-*S*-cysteinyldopa and 23mg (5.8% yield) of 2-*S*-cysteinyldopa as the dihydrochloride salts. The structures of 5-*S*-cysteinyldopa (Prota *et al.*, 1968) and 2-*S*-cysteinyldopa (Fattorusso *et al.*, 1969) were confirmed by their characteristic u.v. spectra and the n.m.r. spectrum (1 M-$^2$HCl) of the former ($\delta$ 6.94 and 7.03, AB-type quartet, $J=2$). $R_F$ values of 5-*S*-cysteinyldopa, 2-*S*-cysteinyldopa and cystine in solvent (1) were 0.30, 0.24 and 0.22 respectively.

2,5-*SS*-*Dicysteinyldopa* was isolated as the hydrochloride salt and then purified as the formate salt as described for the natural sample. The isolated material was an amorphous colourless powder and weighed 41mg (8.5% yield), $[\alpha]_D^{20} = 115°$ (*c* 0.38 in

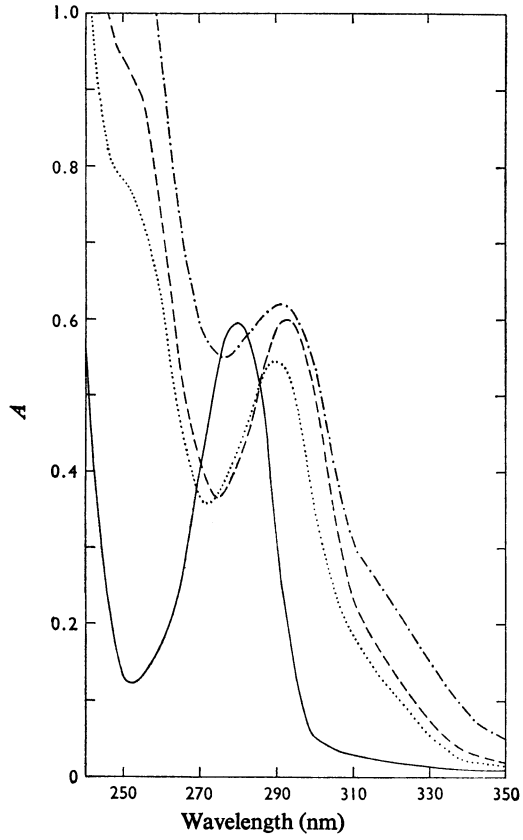



Fig. 5. *Reaction of dopa and cysteine in the presence of tyrosinase*

Samples (0.05ml) of the reaction mixture were added to 1.95ml of 0.05M-sodium phosphate buffer, pH6.8, containing 0.01M-mercaptoethanol, and their u.v. spectra were measured: ——, 0h (before the addition of tyrosinase; $\lambda_{max.}$ 280nm); ····, 3h ($\lambda_{max.}$ 290nm); – – – –, 6h ($\lambda_{max.}$ 293nm); ·–·–, 10h ($\lambda_{max.}$ 292nm). See the Experimental section for further details.

1M-HCl) (Found: C, 39.9; H, 5.2; N, 8.8; S, 13.4; ash, 0.2. $C_{15}H_{21}N_3O_8S_2,HCO_2H$ requires C, 39.9; H, 4.8; N, 8.7; S, 13.3%). The u.v. spectra are shown in Fig. 3 and the n.m.r. spectrum (part) in Fig. 4.

*Enzymic synthesis of* 3-*S-cysteinylcatechol and* 3,6-*SS-dicysteinylcatechol*

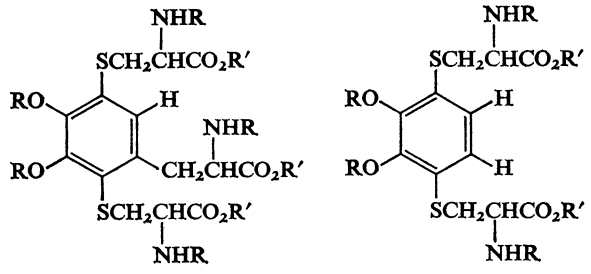
Mushroom tyrosinase (33mg) was added to a solution consisting of 100mg (1mmol) of catechol and 363mg (3mmol) of L-cysteine in 100ml of 0.05M-sodium phosphate buffer, pH6.8, and the solution was stirred at 22°C. The reaction was followed by u.v. spectrophotometry and after 10h was stopped by the addition of 2ml of 6M-HCl. The mixture was passed through a column (2.2cm× 5.7cm) of Dowex-50W (X4, $H^+$ form, 200–400 mesh). The column was eluted with 1000ml of 1M-HCl and then 700ml of 3M-HCl.

Fraction (150–800ml) of the 1M-HCl eluate contained 3-*S*-cysteinylcatechol along with cysteine and cystine. The fraction was evaporated to dryness under reduced pressure and the residue was chromatographed on the Sephadex LH-20 column (described above). Fractions 54–65, which contained 3-*S*-cysteinylcatechol, were combined, evaporated and dried in a vacuum desiccator. The crystalline residue was recrystallized from acetic acid/diethyl ether (over charcoal) to give 118mg (41%) of *3-S-cysteinylcatechol hydrochloride monohydrate* as needles. It softened at 114°C and then melted with decomposition at 172–175°C; i.r. in KBr: $\nu_{max.}$ 3530, 3450, 1755, 1610, 1585 and 1510$cm^{-1}$; u.v. in 0.1M-HCl: $\lambda_{max.}$ 252 and 288nm ($\varepsilon$ 3530 and 2610 litre·$mol^{-1}$·$cm^{-1}$) (Found: after drying at 100°C under high vacuum; C, 40.8; H, 4.6; N, 5.4; S, 12.1; weight loss, 5.3. $C_9H_{11}NO_4S,HCl$ requires C, 40.7; H, 4.6; N, 5.3; S, 12.1; weight loss as the monohydrate salt, 6.3%).

The 3M-HCl eluate, which contained almost exclusively 3,6-*SS*-dicysteinylcatechol, was evaporated to dryness under reduced pressure and dried in a vacuum desiccator over KOH pellets. The crystalline residue was dissolved in 5–6ml of water and 10mg of $NaHSO_3$ was added. The residue was treated with charcoal and the solution adjusted to approx. pH4 with 0.8ml of 1M-sodium acetate. Fine needles were immediately precipitated; these were filtered and washed with water, ethanol and then acetone. Drying gave 65.8mg (17%) of *3,6-SS-dicysteinylcatechol dihydrate*. When heated rapidly, it melted at 262°C (decomp.) in a sealed tube; i.r. in KBr: $\nu_{max.}$ 3600, 1655, 1625, 1600, 1500 and 1400$cm^{-1}$; u.v. in 0.1M-HCl: $\lambda_{max.}$ 212, 270 and 298nm (shoulder) ($\varepsilon$ 26600, 10800 and 3200 litre·$mol^{-1}$·$cm^{-1}$ respectively) (Found: C, 37.6; H, 5.2; N, 7.4; S, 16.8. $C_{12}H_{16}N_2O_6S_2,2H_2O$ requires C, 37.5; H, 5.2; N, 7.3; S, 16.7%.) $R_F$ values of 3-*S*-cysteinylcatechol and 3,6-*SS*-dicysteinylcatechol in solvent (2) were 0.73 and 0.37.

*Preparations of* (*a*) 2,5-*SS-dicysteinyldopa NN'N''-OO'-penta-acetyltrimethyl ester* (*Ib*) *and* (*b*) 3,6-*SS-dicysteinylcatechol NN'OO'-tetra-acetyl dimethyl ester* (*IIb*)

(*a*) Dry HCl gas was passed through a suspension of 25.0mg of synthetic 2,5-*SS*-dicysteinyldopa (*Ia*) in 10ml of methanol until saturation. The resulting solution was left for 2 days at room temperature (about 23°C). Then the solvent was evaporated under reduced pressure and the residue was dried thoroughly in a vacuum desiccator. To the crude methyl ester hydrochloride were added 2.5ml of acetic anhydride and 0.5ml of pyridine. After 2 days at room tempera-



(I*a*) R = R′ = H (II*a*) R = R′ = H

(I*b*) R = $CH_3CO$, R′ = $CH_3$ (II*b*) R = $CH_3CO$, R′ = $CH_3$

Scheme 1.

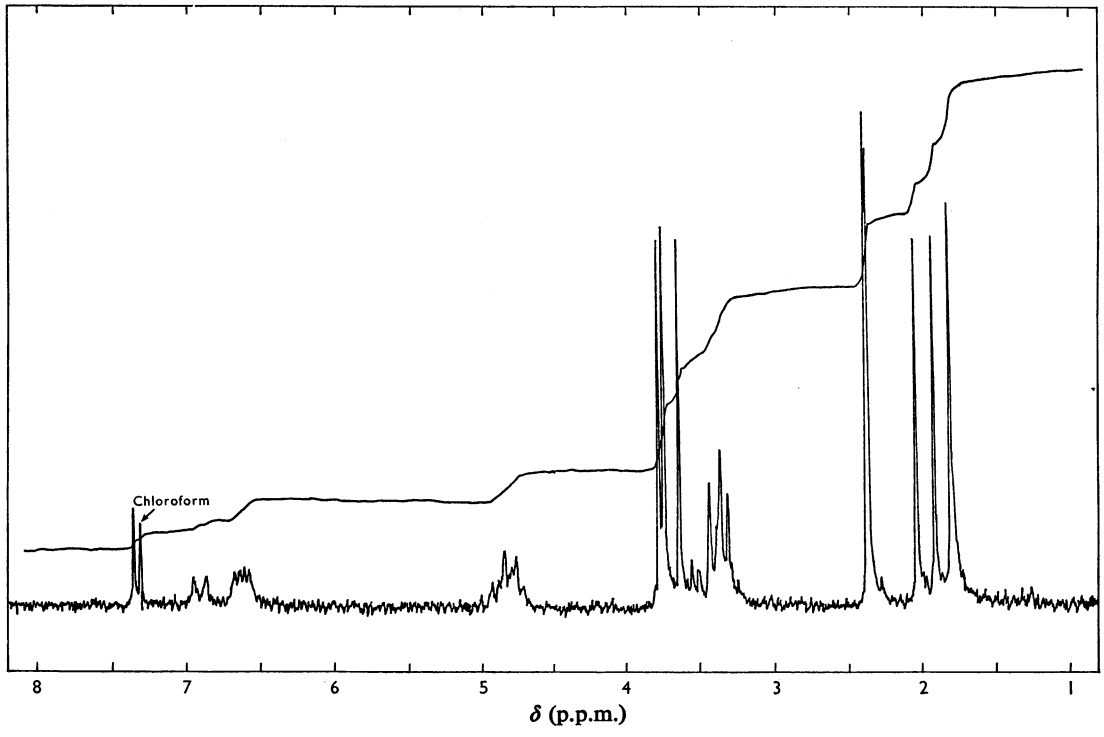



Fig. 6. *N.m.r. spectrum of* 2,5-*SS-dicysteinyldopa NN′N″OO′-penta-acetyl trimethyl ester* (*Ib*)
The spectrum was taken in [$^2$H]chloroform.

ture the mixture was evaporated to dryness under reduced pressure at 50°C. The residue was taken up in 20ml of ethyl acetate and the solution was washed successively with 5ml each of 1M-HCl, water, saturated $NaHCO_3$ solution and water. After drying over $Na_2SO_4$ the ethyl acetate was evaporated and the residue was subjected to preparative t.l.c. on silica gel GF (20cm×20cm, 0.10mm thick) in chloroform/methanol (19:1, v/v). The product (I*b*), 8.0mg (22%), was obtained as a colourless oil; i.r. (neat): $\nu_{max.}$ 1780, 1745, 1660 and 1530$cm^{-1}$; u.v. in methanol: $\lambda_{max.}$ 279nm ($\varepsilon$ 10700 litre·$mol^{-1}$·$cm^{-1}$). The n.m.r. spectrum is shown in Fig. 6; some contamination was evident from the integration of the region $\delta$ 1.7–1.9.

(*b*) 3,6-*SS*-Dicysteinylcatechol (II*a*) (14.2mg) was

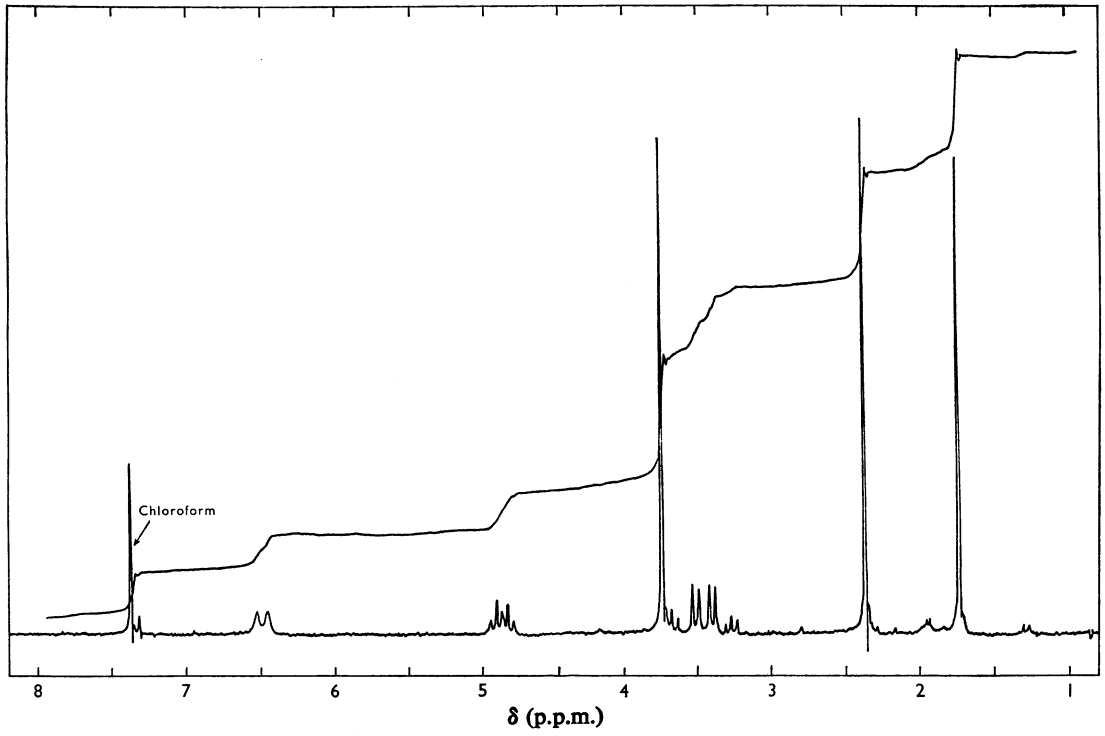



Fig. 7. *N.m.r. spectrum of* 3,6-*SS-dicysteinylcatechol NN'OO'-tetra-acetyl dimethyl ester (IIb)*
The spectrum was taken in [$^2$H]chloroform.

esterified and acetylated as described in (*a*). The product (IIb) (18.0mg, 81%), was obtained as a colourless oil; i.r. (neat): $\nu_{max.}$ 1775, 1745, 1660 and 1530cm$^{-1}$; u.v. in methanol: $\lambda_{max.}$ 277nm ($\varepsilon$ 13200 litre·mol$^{-1}$·cm$^{-1}$). The n.m.r. spectrum is shown in Fig. 7: $\delta$ 1.76 (NCOMe×2), 2.40 (OCOMe×2), 3.37 (one H of $CH_2$×2; doublet of doublets, $J$= 14 and 4), 3.55 (one H of $CH_2$×2; doublet of doublets, $J$ = 14 and 4) 3.76 ($CO_2$Me×2), 4.88 (CH×2; doublet of triplets, $J$ = 7.5 and 4), 6.49 (NH×2; doublet, $J$ = 7.5) and 7.38 (aromatic H×2).

## Results and Discussion

The two-dimensional chromatogram of tapetum extract (Fig. 1) revealed the presence of a number of u.v.-absorbing compounds, among which the compounds (*a–e*) reacted with both ninhydrin and $FeCl_3$ reagents. The compounds (*h*) and (*i*) were identified by t.l.c. as guanine and hypoxanthine respectively. Choroid extract contained guanine and hypoxanthine in quantities about ten times that of tapetum extract. This result indicates that the compounds (*h*) and (*i*) came not from the pigment epithelium, but from the underlying choroid which contains a stratum argentea. It seems that the compounds (*a–e*) [and possibly (*f*) and (*g*)] constitute the reflecting material in the tapetum lucidum of the gar. Among them the compound (*a*) (amino acid A) was apparently the most abundant. Further evidence for localization comes from histological sections in which pigment epithelial cell processes stain intensely with $FeCl_3$/$K_3Fe(CN)_6$ (Nicol & Arnott, 1973).

The amino acid A was successfully isolated as a colourless powder from whole eyes and the yield was 0.51mg/eye or 0.19mg/g wet wt. of eye. It gave, on t.l.c., single spots which migrated at the same rates as the spot (*a*) of tapetum extract. The elementary analysis agreed well with the formula $C_{15}H_{21}N_3O_8S_2$,-$HCO_2H$. The i.r.-absorption spectrum showed broad absorptions at 3600–2200, 1630, 1500 and 1390cm$^{-1}$, characteristic of an $\alpha$-amino acid. The presence of an *o*-diphenolic chromophore was suggested by the green colour with $FeCl_3$ and its extreme instability in an alkaline solution. The pH-dependence of the u.v. spectrum showing an absorption maximum at 303nm in 0.1M-HCl, which was shifted to 316nm at pH6.8 (Fig. 3), was also typical of an *o*-diphenol compound. Besides the formate proton, which appeared at $\delta$ 8.30 as a sharp singlet, the n.m.r.

spectrum (1M-$^2$HCl; Fig. 4) revealed a complex multiplet at $\delta$ 3.2–3.8 for six methylene protons, a complex multiplet at $\delta$ 4.3–4.5 for three methine protons and a sharp singlet (1H) at $\delta$ 7.20 attributable to an isolated aromatic proton.

The amino acid composition of the compound (*a*) was unambiguously determined by reductive hydrolysis with hydriodic acid/red phosphorous (Nicolaus *et al.*, 1969), yielding cysteine and dopa (50% yield), along with another *o*-diphenolic amino acid (20%), which was later identified as 5-*S*-cysteinyldopa. On the other hand, the amino acid A remained almost unchanged when heated in 6M-HCl at 110°C for 24h. A structure in accord with these physical and chemical data is *SS*-dicysteinyldopa constructed from one molecule of dopa and two molecules of cysteine through thio-ether linkages.

Biosynthetically, *SS*-dicysteinyldopa could be formed by consecutive additions of two cysteine molecules to quinones generated by the action of tyrosinase from dopa and then from *S*-cysteinyldopa. We therefore studied tyrosinase oxidation of L-dopa (1equiv.) in the presence of L-cysteine (3equiv.) in the hope that it might give the same amino acid. Spectrophotometric examination of the reaction (Fig. 5) showed that the initial absorption maximum of dopa at 280nm was gradually replaced during 6h by an absorption maximum at 292nm corresponding to 5-*S*-cysteinyldopa and during the next 4h the absorption above 310nm was considerably increased. Three *o*-diphenolic amino acids were isolated by ion-exchange and paper chromatography from the oxidation mixture. Two were identified as 5-*S*-cysteinyldopa (68% yield) and 2-*S*-cysteinyldopa (5.8%), known products of 1,6-addition of cysteine to dopaquinone. The third amino acid (8.5%; $C_{15}H_{21}N_3O_8S_2,HCO_2H$ by elementary analysis), had mobilities on t.l.c. [solvents (2), (5) and (6)] identical with those of the natural amino acid A. Their i.r.- and u.v.-absorption spectra (Fig. 3) were both almost identical with each other, although the latter showed the natural sample to be contaminated to a minor extent. More important still, the n.m.r. spectra (Fig. 4) were superimposable on each other in every detail. Thus the amino acid from the gar and that prepared by the enzymic synthesis were proved to be identical.

The optimum conditions for enzymic synthesis so far achieved are described in the Experimental section. Once all the cysteine added is consumed by incorporation into the products and by air oxidation to cystine, the dopa derivatives produced are rapidly oxidized further, giving rise to cyclization products (Prota *et al.*, 1970). It is therefore essential for a higher yield of the amino acid A to stop the reaction at an appropriate time. The enzymic synthesis of the amino acid A confirmed the gross structure *SS*-dicysteinyldopa for which three structures were possible: 2,5-*SS*-dicysteinyldopa (structure I*a*), 5,6-*SS*-dicysteinyldopa and 2,6-*SS*-dicysteinyldopa. These structures were consistent with the n.m.r. spectrum (Fig. 6) of the *NN'N''OO'*-penta-acetyl trimethyl ester derivative (I*b*) which was prepared by esterification followed by acetylation. The presence of three acetamide, two phenyl acetate and three methyl ester groups was indicated by three singlets ($\delta$ 1.82, 1.94 and 2.04), two singlets (2.37 and 2.38) and three singlets (3.64, 3.74 and 3.78) respectively, and an isolated aromatic proton appeared at $\delta$ 7.34 as a sharp singlet.

Although there was no direct chemical or spectral evidence which favoured any one of the three structures for the amino acid, the fact that the addition of cysteine to dopaquinone takes place at C-5 or C-2 positions giving 5- or 2-*S*-cysteinyldopa seemed to favour the structure 2,5-*SS*-dicysteinyldopa.

Further support came from an experiment using catechol, the simplest symmetrical *o*-diphenol, as a model compound. Tyrosinase oxidation of catechol in the presence of L-cysteine (3equiv.) gave two crystalline *o*-diphenolic amino acids. The major product (41% yield) was characterized as 3-*S*-cysteinylcatechol on the basis of elementary analysis ($C_9H_{11}NO_4S,HCl$), coupled with the n.m.r. spectrum (1M-$^2$HCl) showing three doublets of doublets for three adjacent aromatic protons at $\delta$ 6.86 ($J$ = 8.5 and 7.5), 7.01 ($J$ = 8.5 and 2) and 7.09 ($J$ = 7.5 and 2) and a doublet at $\delta$ 3.50 ($J$ = 5.5; 2H) and a triplet at $\delta$ 4.27 ($J$ = 5.5; 1H) arising from the cysteine residue.

The minor product (17%), analysed for $C_{12}H_{16}N_2O_6S_2,2H_2O$, showed a u.v. spectrum similar to that of the amino acid A. The n.m.r. spectrum (1M-$^2$HCl) indicated that the product had a symmetrical structure; two methylene groups appeared as a doublet at $\delta$ 3.54 ($J$ = 5.6), two methine groups as a triplet at $\delta$ 4.32 ($J$ = 5.6) and two aromatic protons as a sharp singlet at $\delta$ 7.10. Consequently, the product was assigned the structure 3,6-*SS*-dicysteinylcatechol (compound II*a*) derived from two consecutive additions of cysteine, first to 1,2-benzoquinone and then to 3-*S*-cysteinyl-1,2-benzoquinone. The symmetrical structure was also consistent with the n.m.r. spectrum (Fig. 7) of the *NN'OO'*-tetra-acetyl dimethyl ester (II*b*; for details see the Experimental section). Therefore the structure I(*a*), 3-(2,5-*SS*-dicysteinyl-3,4-dihydroxyphenyl)-alanine (2,5-*SS*-dicysteinyldopa), was assigned to the amino acid A, the major constituent of the reflecting material of the eye of the alligator gar.

The absolute configuration of the natural amino acid was inferred as LLL through the good agreement of the specific rotation of the natural sample ($[\alpha]_D$ = +113°) with that of the synthetic one ($[\alpha]_D$ = +115°) which was prepared from L-dopa and L-cysteine. The

biosynthesis of the amino acid also makes probable an L configuration for the dopa moiety.

We have isolated from the tapetum lucidum of the sea catfish *Arius felis* (L.) oligomers of 5,6-dihydroxyindole-2-carboxylic acid which, we believe, may be formed from tyrosine or its equivalent by tyrosinase oxidation (Ito & Nicol, 1974). 2,5-*SS*-Dicysteinyldopa is another reflecting material for which biosynthesis may involve tyrosinase.

It has been shown that phaeomelanins and trichochromes, yellow to reddish pigments of hair and feathers, are formed *in vivo* from tyrosine and cysteine by tyrosinase oxidation (Misuraca *et al.*, 1969; Thomson, 1974) by the intermediacy of 5- and 2-*S*-cysteinyldopa (Prota *et al.*, 1968; Fattorusso *et al.*, 1969; Nicolaus *et al.*, 1969). Although 2,5-*SS*-dicysteinyldopa has not been considered as one of the intermediates in the phaeomelanin synthesis, its enzymic synthesis *in vitro* along with 5- and 2-*S*-cysteinyldopa indicates that it may also participate in the formation of phaeomelanins. In this connexion, it is noteworthy that, in addition to other metabolites arising from dopa and cysteine such as 5- and 2-*S*-cysteinyldopa and trichochromes, 2,5-*SS*-dicysteinyldopa was isolated from the urine of patients suffering from melanoma (G. Prota, H. Rorsman, A. M. Rosengren & E. Rosengren, personal communication). It now seems likely that the phaeomelanin synthesis may take place in the eye of the gar. Further, compounds (*b–e*) [and possibly compounds (*f*) and (*g*)] of tapetum extract (see Fig. 1) may also be metabolites of dopa and cysteine related to phaeomelanins and therefore their structural studies should be of considerable interest.

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