Biochemical Pharmacology, Vol. 36, No. 12, pp. 2007–2011, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

MECHANISM OF SELECTIVE TOXICITY OF 4-S-CYSTEINYLPHENOL AND 4-S-CYSTEAMINYLPHENOL TO MELANOCYTES

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(*Received* 24 *April* 1986; *accepted* 19 *January* 1987)

Abstract—Our previous studies showed that 4-S-cysteinylphenol (4-S-CP) and 4-S-cysteaminylphenol (4-S-CAP) inhibit the growth of malignant melanoma and cause depigmentation of black skin. In this study we examined kinetic constants of CP and CAP as substrates for tyrosinases and their properties as sulphydryl scavengers. 4-S-CP and 4-S-CAP were found to be much better substrates for mushroom tyrosinase than L-tyrosine while their 2-S isomers were not the substrates. 4-S-CP and 4-S-CAP were also good substrates for mammalian tyrosinase. Upon tyrosinase oxidation the two phenols conjugated with cysteine to form the cysteinyl derivatives of the corresponding catechols via *o*-quinone forms. The tyrosinase oxidation product of 4-S-CP had a poor ability to conjugate with alcohol dehydrogenase, a sulphydryl enzyme, while that of 4-S-CAP had a much higher ability. These results suggest that in melanocytes these phenols are oxidised by tyrosinase to the corresponding *o*-quinone forms, some of which conjugate with sulphydryl enzymes through cysteine residues, thus exerting cytotoxic effects.

Many attempts have been made to develop rational chemotherapy for malignant melanoma by taking advantages of its unique biochemical property, melanin synthesis [1]. The synthesis of melanin pigment from L-tyrosine is catalysed by tyrosinase (EC 1.14.18.1) present in both normal and malignant melanocytes. Previously, Wick showed that catecholic compounds related to dopa and dopamine possess significant antitumour activities against mouse and human melanomas *in vitro* and *in vivo* [2–4].

A major drawback in using catechols as chemotherapeutic agents is that they possess certain degrees of systemic toxicity that may result from autoxidation of catechols and concomitant production of active oxygen species [5]. As a result, some catechols exhibit less antimelanoma effect than the corresponding phenols [6]. A rational approach to overcome this difficulty appears to use phenolic compounds that are immediate precursors of catecholic compounds. The phenols may be hydroxylated by tyrosinase to form catechols within melanocytes.

As a possible mechanism of the selective toxicity of catechols to melanoma cells, Graham *et al.* [5, 7] and Wick [8] have posulated that the catechols are oxidised by tyrosinase to the *o*-quinone forms that act as sulphydryl reagents, thus inhibiting the activities of essential enzymes such as DNA polymerase α.

Recently, we synthesized 4-S-cysteinylphenol (4-S-CP¶), a sulphur homologue of tyrosine, and its amine derivative, 4-S-cysteaminylphenol (4-S-CAP), and showed that these phenols increased the life span of melanoma-bearing mice and inhibited the melanoma growth [9]. We also showed that 4-S-CP and 4-S-CAP caused depigmentation of black skin of guinea pigs when applied topically [10] and that [^{3}H]4-S-CP was selectively incorporated to pigmented melanoma cells, but not to non-pigmented melanoma cells [11]. To clarify the mechanism of the selective accumulation and toxicity of 4-S-CP and 4-S-CAP in melanocytes, we have examined their effectiveness as substrates for mushroom and mammalian tyrosinases and their abilities to conjugate with sulphydryl enzymes by tyrosinase oxidation (Fig. 1).

MATERIALS AND METHODS

General. Mushroom tyrosinase (2000 units/mg), yeast alcohol dehydrogenase (ADH), L-dopa, and L-cysteine were purchased from Sigma Chemical Co., and the other chemicals were from Wako Pure Chemicals, Ltd. Mammalian tyrosinase was prepared from Harding-Passey melanomas [12]. 4-S and 2-S isomers of CP and CAP were prepared by the reaction of phenol with cystine or cystamine in 47% HBr [9] 4-S-Cysteinylcatechol (4-S-CC) was prepared from pyrocatechol and cystine as described in Ito *et al.* [13]. 4-S-Cysteaminylcatechol (4-S-CAC)

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¶ Abbreviations used: ADH, alcohol dehydrogenase; CAC, cysteaminylcatechol; CAP; cysteaminylphenol; CC, cysteinylcatechol; CD, cysteinyldopa; CP, cysteinylphenol; HPLC, high-performance liquid chromatography.

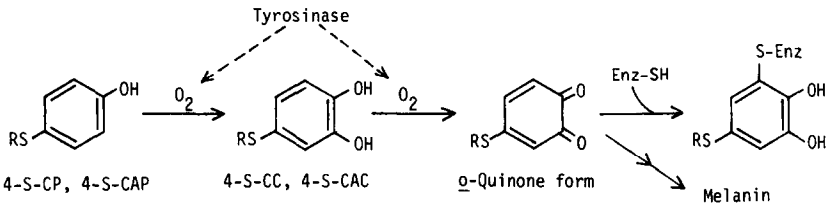

Fig. 1. Possible mechanism of toxicity of 4-S-CP and 4-S-CAP to melanocytes. R = $-CH_2CH(NH_2)COOH$ or $-CH_2CH_2NH_2$.

was similarly prepared from pyrocatechol and cystamine and was separated from the 2-S isomer by chromatography on Dowex 50W. 4-S-CAC thus obtained was crystalline as the HCl salt which could not be recrystallised, but was proved to be homogeneous by high-performance liquid chromatography (HPLC).

NMR spectra were taken with a JEOL FX90QE NMR spectrometer using as an internal reference the methyl signal of 2-methyl-2-propanol which appeared at δ 1.28. HPLC was carried out with a Yanaco Liquid Chromatograph under the conditions used for the cysteinyldopa (CD) determination [14].

Preparation of melanins from 4-S-CP and 4-S-CC. A solution of 4-S-CP (0.2 mmol) or 4-S-CC (0.2 mmol) in 80 ml of 0.05 M sodium phosphate buffer, pH 6.8, was incubated at 37° in the presence of 2 mg of mushroom tyrosinase. After 4 hr the dark violet solution was acidified to pH 1 by adding 2 ml of 6 M HCl. After standing for 1 hr at 4°, the dark violet precipitate was collected by centrifugation, washed three times with 10 ml of 0.1 M HCl, and dried in a desiccator over P_2O_5 and NaOH. 4-S-CP and 4-S-CC gave 21.9 mg and 29.0 mg of melanin-like pigments, respectively. 4-S-CP-melanin: Anal. Found: C, 42.66; H, 3.58; N, 6.86; S, 13.04%. 4-S-CC-melanin: Anal. Found: C, 42.47; H, 3.23; N, 6.70; S. 13.65%.

Determination of kinetic constants. A reaction mixture contained a substrate at 8 different concentrations (0.06–1.0 mM for mushroom tyrosinase and 0.04–0.3 mM for mammalian tyrosinase) and either 6.7 μg of mushroom tyrosinase or 1.4 μg of mammalian tyrosinase in 1 ml of 0.05 M sodium phosphate buffer, pH 6.8. In case of mammalian tyrosinase, 10 μM L-dopa was added as a cofactor. The reaction was carried out at 37°, and dopachrome(-type) pigment formation was followed at 475 nm (ε 3700) for L-tyrosine [15], 570 nm for 4-S-CP or 565 nm for 4-S-CAP. When mammalian tyrosinase was used, the pigment formation was followed at 600 nm to avoid the influence of dopachrome. Molar extinction coefficients (ε) were determined with the reaction mixture containing 0.1 mM substrate (4-S-CP or 4-S-CAP) and 50 μg of mushroom tyrosinase in 1 ml of the pH 6.8 buffer. 4-S-CP and 4-S-CAP yielded violet pigments showing absorption maxima at 375 nm (ε 17,000) and 570 nm (ε 1250) for 4-S-CP and 370 (ε 17,500) and 565 nm (ε 1050) for 4-S-CAP. K_m and V_{max} values were calculated from the Lineweaver-Burk plots.

Tyrosinase-mediated conjugation of 4-S-CC with cysteine (preparative). A solution of 0.5 mmol of 4-S-CC and 1.0 mmol of L-cysteine in 50 ml of 0.05 M sodium phosphate buffer, pH 6.8, was stirred at room temperature (24°) in the presence of 25 mg of mushroom tyrosinase. The reaction was monitored by u.v. spectrophotometry. After 2 hr when an absorption maximum at 288 nm due to 4-S-CC was replaced by a new maximum at 300 nm, the reaction was stopped by adding 5 ml of 6 M HCl. The reaction mixture was passed through a column (2.0 × 8 cm) of Dowex 50W-X2 (200–400 mesh; equilibrated with water). After washing with 100 ml of 0.5 M HCl, the products were eluted with 3 M HCl, and 20-ml fractions were collected and monitored spectrophotometrically. Fractions 3–19 that contained dicysteinylcatechol were combined and evaporated to dryness in a rotary evaporator. The residue, taken up in 2 M HCl, was rechromatographed on a column (2.0 × 18 cm) of Dowex 50W-X2 (200–400 mesh, equilibrated with 2 M HCl), and 20-ml fractions were collected and monitored. A major peak appeared in fractions 60–96 which were combined and evaporated in a rotary evaporator. The residue was dried in a desiccator over P_2O_5 and NaOH. A mixture of isomers of S,S'-dicysteinylcatechol·2HCl was obtained as a glassy solid in a yield of 188 mg (90% yield). The HCl salt was converted to the free form by dissolving it in 5 ml of 1% sodium metabisulfite and adding sodium acetate to pH *ca.* 5. Colorless crystals of 3,5-S,S'-dicysteinylcatechol (99% purity by HPLC) were obtained in a yield of 55 mg (31%): mp 250° dec; u.v. (0.1 M HCl) λ_{max} 248 nm (ε 9530) and 300 nm (2680); ^1H NMR (2 M DCl – D_2O) δ 3.52 (4H, br d, J = 5 Hz, CH_2 × 2), 4.29 (2H, br t, J = 5 Hz, CH × 2), 7.15 and 7.27 (2H, ABq, J = 2.2 Hz). Anal. Calcd for $C_{12}H_{14}N_2O_6S_2 \cdot 0.5H_2O$: C, 40.33; H, 4.79; N, 7.84; S, 17.94%. Found: C, 40.69; H, 4.58; N, 7.83; S, 17.55%.

Tyrosinase-mediated conjugation of 4-S-CAC with cysteine (preparative). The preparation was carried out under similar conditions as described for 4-S-CC (see above). The second chromatography was done on a column (2.0 × 16 cm) of Dowex 50W-X2 (200–400 mesh; equilibrated with 3 M HCl). 5-S-Cysteaminyl-3-S'-cysteinylcatechol·2HCl was obtained in a yield of 70 mg (37%) as a glassy solid by evaporation of fractions 75–100. The u.v. spectrum (λ_{max} 245 and 300 nm) closely resembled that of 3,5-S,S'-dicysteinylcatechol. The purity was *ca.* 90% as determined by HPLC. The yield of the cysteine conjugate

Table 1. Kinetic data for CP and CAP as substrates for tyrosinase*

Substrate	Mushroom tyrosinase		Mammalian tyrosinase†	
	K_m (μM)	V_{max} (μmol/min/mg)	K_m (μM)	V_{max} (μmol/min/mg)
L-Tyrosine	330	1.8	Not determined	
4-S-CP	93	5.7	21	2.3
2-S-CP	—	0.0	—	0.0
4-S-CAP	96	12	31	2.3
2-S-CAP	—	0.0	—	0.0

* Values were obtained from the means for duplicate analyses.
† Determined in the presence of 10 μM L-dopa.

in the reaction mixture was as high as 80%, but the isolated yield was rather low due to autoxidation.

RESULTS

4-S-CP and 4-S-CAP as substrates for tyrosinases

Tyrosinase oxidation of 4-S-CP gave a violet pigment showing absorption maxima at 375 and 570 nm. The pigment may be the sulphur homologue of dopachrome on the basis that the same pigment was also produced from 4-S-CC by tyrosinase oxidation and that it had a spectral pattern similar to that of dopachrome, although with marked bathochromic shifts of the absorption maxima. 4-S-CAP yielded a pigment whose absorption spectrum was almost identical to that of the 4-S-CP pigment.

The fate of the violet pigment from 4-S-CP was next examined. Tyrosinase oxidation of 4-S-CP on a preparative scale gave a dark-violet melanin. The melanin was soluble at pH 6.8 but insoluble at pH 1. The elemental analysis showed the molar ratio of C:N:S to be 7.25:1.00:0.83. A melanin with a similar elemental composition was also obtained from 4-S-CC.

Table 1 summarizes kinetic data obtained with CP and CAP as substrates for mushroom and mammalian tyrosinases. 4-S-CP and 4-S-CAP were much better substrates for mushroom tyrosinase than was L-tyrosine with respects to both K_m and V_{max} values. At a 1 mM concentration, both 4-S-CP and 4-S-CAP exhibited a substrate inhibition of the oxidation as did L-tyrosine. 2-S isomers were not oxidised by mushroom tyrosinase even at a 50 μg/ml concentration.

4-S-CP and 4-S-CAP were not oxidised by mammalian tyrosinase when a cofactor was not added. In the presence of 10 μM L-dopa, however, these phenols acted as substrates for mammalian tyrosinase with markedly low k_m values. At concentrations higher than 0.2 mM, both phenols showed a strong substrate inhibition. 2-S isomers were not oxidised by mammalian tyrosinase in the presence of 10 μM L-dopa.

Tyrosinase-mediated conjugation of 4-S-CP and 4-S-CAP with cysteine

In preliminary experiments, it was found that cysteine suppressed tyrosinase oxidation of 4-S-CP and 4-S-CAP to rates too slow for preparative purpose. Therefore, the oxidation was performed with the catechol derivatives 4-S-CC and 4-S-CAC. Tyrosinase oxidation of 4-S-CC in the presence of cysteine gave a mixture of isomers of S,S′-dicysteinylcatechol in 90% yield. A major isomer was purified by crystallisation. The compound was identified as 3,5-S,S′-dicysteinylcatechol on the basis of an elemental analysis and a NMR signal showing meta substitution on the aromatic ring: two proton signals at δ 7.15 and 7.22 with a coupling constant of 2.2 Hz. Similarly, 4-S-CAC reacted with cysteine by tyrosinase oxidation to form 5-S-cysteaminyl-3-S′-cysteinylcatechol. The cysteine conjugate was identified by the similarity of the u.v. spectrum to that of 3,5-S,S′-dicysteinylcatechol.

Next, analytical experiments were performed. The tyrosinase-mediated conjugation of 4-S-CP and 4-S-CAP with cysteine were compared with that of L-tyrosine. The reactivity of 4-S-CC was also compared with that of L-dopa. As shown in Table 2, whereas L-tyrosine remained mostly unchanged, 4-S-CP and 4-S-CAP were oxidised to significant extents by mushroom tyrosinase to form the cysteine conjugates. Similarly, while only 56% of L-dopa was oxidised, 4-S-CC was almost completely consumed to form the cysteine conjugates. These results indicate that upon tyrosinase oxidation, 4-S-CP and 4-S-CAP conjugate with cysteine after conversion to the corresponding catechols (and then to the *o*-quinone forms) and that 4-S-CP and 4-S-CAP are oxidised much faster than L-tyrosine.

Tyrosinase-mediated conjugation of 4-S-CP and 4-S-CAP with ADH through cysteine residues

We have recently shown that upon tyrosinase oxidation L-dopa bind covalently with proteins through cysteine residues to form protein-bound CD, the yield of which varies greatly depending upon the reactivity of the cysteine residues [17]. Therefore, the tyrosinase-mediated conjugation of 4-S-CP and 4-S-CAP with ADH through cysteine residues was examined in comparison with that of L-tyrosine. The reactivity of 4-S-CC was also compared with that of L-dopa.

As shown in Table 3, great differences were observed in the ability of the phenols to combine with ADH through cysteine residues. Although both 4-S-CP and 4-S-CAP were oxidised at comparable rates, the degree of conjugation of 4-S-CP was only 3.6%, while that of 4-S-CAP was much higher, 17%.

Table 2. Tyrosinase-mediated conjugation of phenols and catechols with cysteine*

Substrate	Substrate oxidised (μM)	Products formed (μM)
L-Tyrosine	3	Dopa 0, 5-S-CD 1
4-S-CP	25	4-S-CC 0, 3,5-S,S'-Dicysteinylcatechol 21†
4-S-CAP	73	4-S-CAC 0, 5-S-Cysteaminyl-3-S'-cysteinylcatechol 50†
L-Dopa	56	5-S-CD 45, 2-S-CD 8, 6-S-CD 1, 2,5-S,S'-Dicysteinyldopa 2‡
4-S-CC	98	3,5-S,S'-Dicysteinylcatechol 54†

* The reaction mixture contained 100 μM substrate, 200 μM L-cysteine and 5 μg of mushroom tyrosinase in 1 ml of 0.05 M sodium phosphate buffer, pH 6.8. The oxidation was carried out at 37° for 5 min and stopped by adding 9 vol. of 0.4 M $HClO_4$. The mixture was directly analysed by HPLC. Values are means for two separate experiments.

† Other products, most likely the isomers, were also detected.

‡ In a previous preparative experiment [16], the yields of 5-S-CD, 2-S-CD, 6-S-CD and 2,5-S,S'-dicysteinyldopa were 74, 14, 1 and 5%, respectively.

Table 3. Tyrosinase-mediated conjugation of phenols and catechols with ADH through the sulphydryl group*

Substrate	Substrate oxidised (μM)	Bound cysteinylcatechols formed† (μM)	Bound cysteinylcatechols/ substrate oxidised (%)
L-Tyrosine	2	5-S-CD + 2-S-CD + 6-S-CD 0.35	17‡
4-S-CP	59	3,5-S,S'-Dicysteinylcatechol 2.1§	3.6
4-S-CAP	65	5-S-Cysteaminyl-3-S'-cysteinylcatechol 11.0§	17
L-Dopa	53	5-S-CD + 2-S-CD + 6-S-CD 18.0	35
4-S-CC	92	3,5-S,S'-Dicysteinylcatechol 4.3§	4.7

* The reaction mixture contained 100 μM substrate, 200 μM ADH and 5 μg of mushroom tyrosinase in 1 ml of 0.05 M sodium phosphate buffer, pH 6.8. The oxidation was carried out at 37° for 5 min and stopped by adding 1 ml of 10% trichloroacetic acid. The supernatant was analysed for the substrate remaining and the precipitate was subjected to the acid hydrolysis to liberate the bound cysteinylcatechols [17]. Values are means for two separate experiments.

† Corrected for the recoveries in the hydrolysis: 5-S-CD 89%; 3,5-S,S'-dicysteinylcatechol, 87%; 5-S-cysteaminyl-3-S'-cysteinylcatechol, 98%. With 5 μg of heat-denatured tyrosinase, the yields of bound cysteinylcatechols were negligible (<0.1 μM).

‡ This value is subjected to a considerable error, as it was calculated on the basis of the low conversion (2.1%) of L-tyrosine.

§ Other products, most likely the isomers, were also detected.

As it was found that the majority of 4-S-CP and 4-S-CAP escaped the conjugation with ADH, the fate of the phenols that did not conjugate with ADH was spectrophotometrically examined. The results showed that the products immediately formed were the violet, dopachrome-type pigments (see above). L-Dopa also conjugated with ADH in 35% yield, a result being similar to the previous one [17]. On the other hand, 4-S-CC had a much lower ability to conjugate with ADH.

From these results, it appears that although both 4-S-CP and 4-S-CAP are equally effective as substrates for tyrosinase, their abilities to bind covalently with proteins are quite different, 4-S-CAP having a much higher ability.

DISCUSSION

Catechol derivatives have been utilised to develop rational chemotherapy of malignant melanoma. The catechols do not, however, necessarily possess anti-melanoma effect *in vivo*, though they possess strong cytotoxicity to melanoma cells *in vitro*. On the contrary, phenolic compounds should be more stable than the catecholic compounds but may become toxic only after oxidation by tyrosinase, an enzyme specific for melanocytes. The present study has shown that 4-S-CP and 4-S-CAP are much better substrates for mushroom tyrosinase than is L-tyrosine and are also good substrates for mammalian tyrosinase in the presence of L-dopa as a cofactor. The requirement of a catecholic cofactor is an essential feature of mammalian tyrosinase [18, 19].

For phenolic compounds to become cytotoxic upon tyrosinase oxidation, not only the oxidation to *o*-quinone forms but also the conjugation with sulphydryl enzymes is required [5, 7, 8]. As a model reaction, we studied the tyrosinase-mediated conjugation of 4-S-CP and 4-S-CAP with cysteine and showed that these phenols have much higher abilities to conjugate with cysteine than L-tyrosine. Further, we showed that while 4-S-CP has a rather poor ability to conjugate with cysteine residues in ADH, a sulphydryl enzyme, 4-S-CAP has an ability 5 times

that of 4-S-CP and a half that of L-dopa. Our previous results that 4-S-CAP has both antimelanoma and depigmenting effects much higher than 4-S-CP [9, 10] may be ascribed to the difference in their abilities to combine with sulphydryl enzymes. The present results suggest that in melanocytes 4-S-CP and 4-S-CAP are oxidised by tyrosinase to the *o*-quinone forms via the catechol derivatives and some of the quinones conjugate with proteins through cysteine residues, thus exerting cytotoxic effects (Fig. 1). The quinones that escape the conjugation eventually polymerise to form melanin-like pigments. Thus, we provided a rational background for the selective accumulation and toxicity of the phenols 4-S-CP and 4-S-CAP in malignant melanoma.

Finally, while 4-S-CP hardly conjugates with proteins, it does effectively conjugate with free sulphydryl compounds. Thus, it might be possible that 4-S-CP selectively depletes melanoma cells of glutathione and thence sensitises them to alkylating agents such as the nitrogen mustards [20].

Acknowledgement—This study was supported by Grant-in-Aid for Cancer Research, No. 5901027 and 6001031 from the Ministry of Education, Science and Culture.

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