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METABOLISM OF 1-NAPHTHOL BY TYROSINASE

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Abstract—1-Naphthol was metabolized by the polyphenol oxidase, tyrosinase, primarily to 1,2-naphthoquinone and to small amounts of 1,4-naphthoquinone as well as to covalently bound products. The inhibition of covalent binding by ethylenediamine, which reacts specifically with 1,2-naphthoquinone but not 1,4-naphthoquinone, suggested that most of the covalent binding was due to 1,2-naphthoquinone or a metabolite of similar structure. The activation by tyrosinase of 1-naphthol to covalently bound products suggested that it may alter the reaction kinetics of the enzyme. This was investigated by studying the effects of 1-naphthol on the tyrosinase-catalysed oxidation of 4-hydroxyanisole. Preincubation of tyrosinase with 1-naphthol increased the lag period of the oxidation of 4-hydroxyanisole, which may be due to a decrease in the amount of active enzyme, as well as to a reaction of 1-naphthol with 3,4-anisylquinone, an oxidation product of 4-hydroxyanisole.

The metabolic activation of 1-naphthol by tyrosinase to covalently bound species suggests that 1-naphthol or a structurally related derivative may be of potential therapeutic application in the treatment of cells high in tyrosinase activity, such as certain melanomas.

The possible therapeutic use of melanocytotoxic chemicals for treatment of malignant melanoma has recently aroused considerable interest. The selectivity of these compounds appears to reside in their metabolic activation by the enzyme tyrosinase (monophenol mono-oxygenase, polyphenoloxidase, catechol oxidase, monophenol-dihydroxyphenylalanine: oxygen oxidoreductase, EC1.14.18.1) to cytotoxic metabolites. Tyrosinase is widely distributed in nature, but in mammals the active form is associated with a specialized cytoplasmic granule, the melanosome, which is present in melanocytes. Tyrosinase catalyses oxidative steps in melanogenesis, e.g. the conversion of tyrosine to dopa quinone. Quinones are highly reactive compounds which undergo additive reactions, especially with thiol or amino groups, and might be considered to be potentially toxic to the cells generating them. Thus, the generation of orthoquinones, either from endogenous or exogenous substrates, may be of potential therapeutic benefit. Tyrosinase is able to oxidize a range of phenolic substrates to produce the corresponding quinones [1, 2] and this mechanism has been proposed for the selective destruction of melanocytes by certain substituted phenols which result in depigmentation of the skin [3, 4]. Recently it has been shown that 4-hydroxyanisole, a substrate for tyrosinase, causes regression of Harding–Passey melanomas transplanted into mice [5] and in initial clinical studies has caused regression of localized malignant melanoma [6, 7].

Recently we have shown that 1-naphthol is selectively toxic to short-term organ cultures of human colonic tumour tissue compared to normal colon from the same patients and therefore may be of value in the treatment of colorectal cancer [8]. In rat liver microsomal preparations, 1-naphthol is metabolized in an NADPH-dependent reaction by the microsomal cytochrome P-450 mixed function oxidase, to 1,4-naphthoquinone and covalently bound species [9]. The major binding species are derived from 1,4-naphthoquinone but not 1,2-naphthoquinone [9]. The toxicity of 1-naphthol to isolated hepatocytes [10] is most probably due to the formation of such cytotoxic naphthoquinones, which may either redox cycle or covalently bind to and modify critical cellular macromolecules.

In this study we show that 1-naphthol may be metabolized by tyrosinase primarily to 1,2-naphthoquinone and to covalently bound products.

MATERIALS AND METHODS

Materials. 1-Naphthol was obtained from the Sigma Chemical Co. Ltd. (Poole, Dorset) or Aldrich Chemical Co. Ltd. (Gillingham, Kent). 1,2- and 1,4-Naphthoquinone were obtained from Fluka (Switzerland) or Sigma Chemical Co. Mushroom tyrosinase (grade 3, 2200–2600 units/mg solid), dimethylsulphoxide (DMSO), NADPH and ethylenediamine dihydrochloride were all purchased from Sigma Chemical Co. HPLC grade methanol was obtained from Rathburn Chemicals Ltd. (Peebleshire, Scotland). 4-Hydroxyanisole (4-HA) was obtained from Koch Light Ltd. (Colnbrook, Bucks.) and recrystallized from water before use. 3,4-Anisylquinone was kindly supplied by Mr C. J. Cooksey. [1-^{14}C]-1-Naphthol (S.A. 19.2 Ci/mole) was obtained from the Amersham International Radiochemical Centre (Amersham, Bucks.).

Metabolism of [1-^{14}C]-1-naphthol to methanol-soluble and covalently bound products. [1-^{14}C]-1-Naphthol (50–500 μM) was incubated with tyrosinase (0.1–2.0 mg/ml) and NADPH (4 mg/ml) in a shaking

water bath at 33° in 0.12 M Tris–HCl, pH 7.4 [11]. At various times, the reaction was stopped by the addition of 2 vols of ice-cold methanol containing unlabelled 1-naphthol, 1,2- and 1,4-naphthoquinone as authentic standards. The protein was removed by centrifugation at 2000 *g* for 5 min and stored at −20° for analysis of covalent binding as previously described [9, 12]. Protein concentration was determined by the method of Lowry *et al.* [13]. The supernatant fraction was analysed by HPLC on an Altex ODS Ultrasphere column (15 cm) for methanol-soluble metabolites [9].

Spectrophotometric investigation of the effects of 1-naphthol and 1,2-naphthoquinone on the induction period of the oxidation of 4-hydroxyanisole. 1-Naphthol or 1,2-naphthoquinone was dissolved in dimethylsulphoxide (DMSO). The reaction mixtures consisted of mushroom tyrosinase (15 μg/ml), 4-hydroxyanisole (100 μM) in 0.1 M phosphate buffer (pH 7.4) to a total volume of 3 ml and was incubated in a spectrophotometer cuvette at 33°. The added material was made up in concentrations enabling 10 μl to be added to give the final concentration. The reaction was monitored by noting the extinction at 260 nm, using a Unicam SP800 spectrophotometer. The induction period is the time elapsed between the beginning of the incubation and the attainment of the maximum reaction velocity and was measured by the extinction at 260 nm. Results were expressed as a proportion of the induction period of the control incubations containing the solvent only.

High-pressure liquid chromatographic investigations of the interaction of 1-naphthol with 3,4-anisylquinone. HPLC analysis was performed on a Pye Unicam (Cambridge, U.K.) system consisting of an LC-XPS pump, an LC-UV detector and a rheodyne 7010 injector. A 250 × 4.6 mm internal diameter Spherisorb ODS HPLC column (5 μ particle size) was employed. A 30/70 (v/v) solution of acetonitrile/phosphate buffered saline was used as the mobile phase and the absorbance monitored at 254 nm. The experiments were made on sample sizes of 20 μl and the elution rate was 1 ml/min. Under these conditions the retention times of 4-hydroxyanisole, 3,4-anisylquinone, and 1-naphthol were 5, 9.25 and 45 min respectively. Incubations were made at room temperature and samples injected at various time points. Concentrations of 4-hydroxyanisole and its quinone were estimated by measuring peak heights.

RESULTS

Metabolism of 1-naphthol by tyrosinase

[1-^{14}C]-1-Naphthol was metabolized by tyrosinase, in the presence of NADPH, to both methanol-soluble metabolites and covalently bound products. High pressure liquid chromatography of the methanol-soluble products showed that the major metabolite co-chromatographed with 1,2-naphthoquinone and a small amount of radioactivity also co-chromatographed with 1,4-naphthoquinone (Fig. 1). The

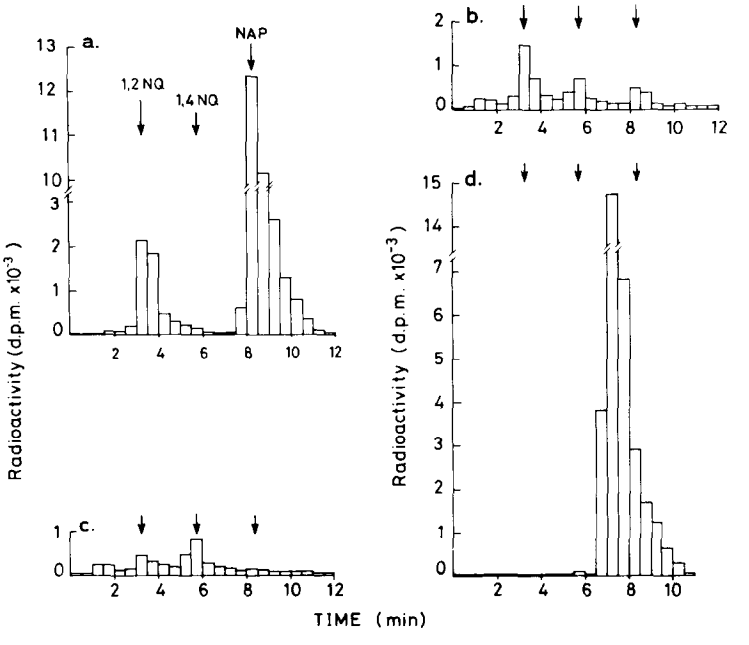


Fig. 1. HPLC separation of metabolites of [1-^{14}C]-1-naphthol catalysed by tyrosinase. [1-^{14}C]-1-Naphthol (500 μM) was incubated with tyrosinase (2 mg/ml) and NADPH (4 mg/ml) at 33° in 0.12 M Tris–HCl pH 7.4 for (a) 5 min, (b) 30 min, (c) 60 min and (d) 60 min in the absence of tyrosinase. The reaction was terminated by the addition of ice-cold methanol containing unlabelled 1-naphthol, 1,2- and 1,4-naphthoquinone. The protein was removed by centrifugation and the supernatant solution analysed by HPLC as described in Materials and Methods. 1-Naphthol (NAP), 1,2-naphthoquinone (1,2NQ) and 1,4-naphthoquinone (1,4NQ) eluted at the times indicated by the arrows. The results shown are from one experiment typical of four.

time course of metabolite production was complex: the radioactivity, which co-chromatographed with 1,2-naphthoquinone, increased initially (Fig. 1a) but then, at longer incubation times such as 30 and 60 min (Fig. 1b and 1c), decreased, most probably due to its covalent binding. In contrast, the radioactivity associated with 1,4-naphthoquinone increased slightly with time (Fig. 1). The metabolism showed a total dependence on tyrosinase, no metabolites being observed in its absence (Fig. 1d). The metabolism of [1- ^{14}C]-1-naphthol (500 μM) was also markedly dependent on the concentration of tyrosinase (0.10–2.0 mg/ml). After 30 min incubation with tyrosinase ($\geq$0.5 mg/ml) almost all the 1-naphthol was metabolized and the major methanol-soluble metabolite co-chromatographed with 1,2-naphthoquinone but small amounts of radiolabel also eluted with 1,4-naphthoquinone. With lower concentrations of tyrosinase (0.1–0.2 mg/ml) significant amounts of 1-naphthol remained unmetabolized together with detectable amounts of both naphthoquinones.

Covalently bound products

Particularly significant was the very marked disappearance of methanol-soluble radioactivity following incubation of [1- ^{14}C]-1-naphthol with tyrosinase, which can be readily appreciated from Fig. 1 (compare Figs 1a and 1c), where equal volumes of the methanol-soluble products were analysed by HPLC. At later times, most of the radioactivity was found to be associated with the protein precipitate formed following the addition of methanol to the sample prior to HPLC analysis. It was concluded that a metabolite of [1-^{14}C]-1-naphthol was covalently bound as the radioactivity was still associated with the protein despite exhaustive washing with organic solvents as described in Materials and Methods. In the presence of NADPH, almost all of the [1-^{14}C]-1-naphthol (500 μM) was covalently bound to tyrosinase (2 mg/ml) following incubation at 33° for greater than 30 min. A marked delay was observed prior to the dramatic increase in covalent binding (Fig. 2). This delay varied in different experiments and may have been due to different batches of tyrosinase. When NADPH was omitted from the reaction mixture, no delay period was observed and all the [1-^{14}C]-1-naphthol was converted to metabolites, which were covalently bound to the tyrosinase at the first time point measured (Fig. 2). In the absence of NADPH, a purple colour developed rapidly in the incubation mixture which was subsequently shown to be associated with the protein. The purple colour was also observed in the incubations with NADPH but in these experiments it only appeared at later times (>20 min).

In a previous study [9] we showed that ethylenediamine reacts specifically with 1,2-naphthoquinone but not 1,4-naphthoquinone. Inclusion of ethylenediamine (20 mM) in the incubation mixture caused a 42% decrease in the covalent binding of [1-^{14}C]-1-naphthol (500 μM) to tyrosinase (2 mg/ml) in the presence of NADPH. As the covalent binding of naphthol to tyrosinase would be expected to alter its reaction kinetics, we examined the effects of 1-naphthol on the kinetics of a well-characterized substrate, 4-hydroxyanisole.

Effect of 1-naphthol on the kinetics of tyrosinase-catalysed oxidation of 4-hydroxyanisole

Pre-incubation of tyrosinase with 1-naphthol increased the induction (or lag) period of the oxidation of 4-hydroxyanisole by tyrosinase as estimated by the formation of the first spectrophotometrically identifiable product (3,4-anisylquinone) (Fig. 3). An increase in the lag phase of tyrosinase action is characteristic of an increase in the relative substrate excess (i.e. the ratio of the molar concentration of substrate to enzyme), which in these experiments would be the consequence of a diminution in the amount of active enzyme. Known competitive inhibitors of tyrosinase, such as 4-nitrophenol [2], do not increase the induction period. As 1,2-naphthoquinone was the main initial oxidation product of 1-

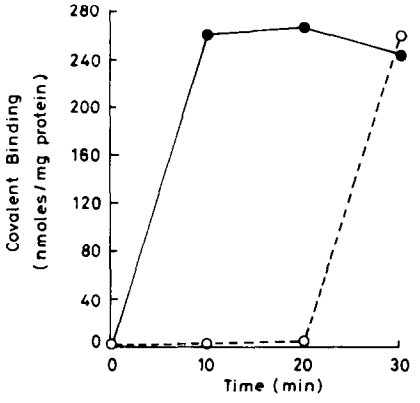


Fig. 2. Covalent binding of [1-^{14}C]-1-naphthol catalysed by tyrosinase in the presence (○---○) or absence (●——●) of NADPH (4 mg/ml). [1-^{14}C]-1-Naphthol (500 μM) was incubated with tyrosinase (2 mg/ml). The protein was isolated and covalent binding determined as described in Materials and Methods. The results shown are from one experiment typical of two.

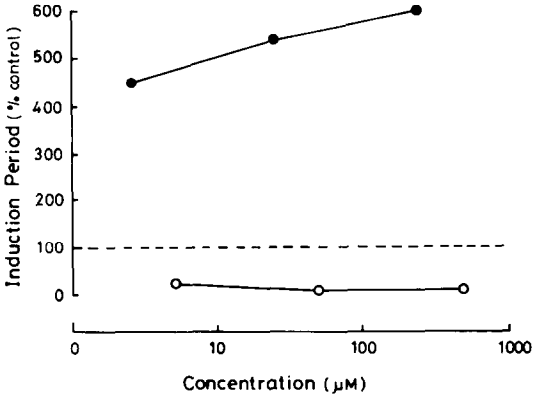


Fig. 3. Effects of varying concentrations of 1-naphthol (●——●) or 1,2-naphthoquinone (○——○) on the induction period of the oxidation of 4-hydroxyanisole by tyrosinase. Tyrosinase (15 μg/ml) was pre-incubated for 10 min with 1-naphthol or 1,2-naphthoquinone in DMSO, or solvent alone (10 μl), in the absence of substrate (4-hydroxyanisole).

naphthol (Fig. 1), we wished to investigate whether this metabolite was responsible for the increase in induction period. Using the spectrophotometric assay, we were unable to demonstrate any increase in the induction period of 4-hydroxyanisole oxidation by tyrosinase (Fig. 3). Indeed, the addition of 1,2-naphthoquinone (5–500 μM) actually shortened the lag period (Fig. 3) in comparison to the appropriate controls. This was not due to a solvent-induced artefact since the solvent (DMSO) had no effect on the reaction kinetics.

With higher concentrations of tyrosinase (50 μg/ml), no induction period was observed in the oxidation of 4-hydroxyanisole. Under these conditions, 1-naphthol caused no inhibition of the overall rate at which the substrate (4-hydroxyanisole) was removed as measured by HPLC but a substantial alteration in the rate of appearance of 3,4-anisylquinone was noted. Under these conditions, in control incubations, after 10 min approximately 60% of the substrate was converted to 3,4-anisylquinone (Fig. 4a). After this time the concentration of 3,4-anisylquinone decreased most probably due to polymerization reactions (Fig. 4a). In the presence of 1-naphthol, an initial maximum formation of 3,4-anisylquinone (about 40% of the initial substrate concentration) was observed followed by a decrease

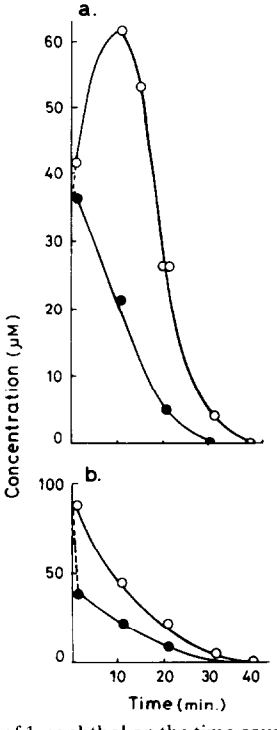


Fig. 4. Effects of 1-naphthol on the time course of oxidation of 4-hydroxyanisole by tyrosinase: (a) time course of appearance of 3,4-anisylquinone in the absence (○——○) or presence (●——●) of 1-naphthol (100 μM); (b) time course of disappearance of 4-hydroxyanisole in the absence (○——○) or presence (●——●) of 1-naphthol. 4-Hydroxyanisole (100 μM) was incubated with tyrosinase (50 μg/ml) and both the substrate and the initial oxidation product, 3,4-anisylquinone measured by HPLC as described in Materials and Methods.

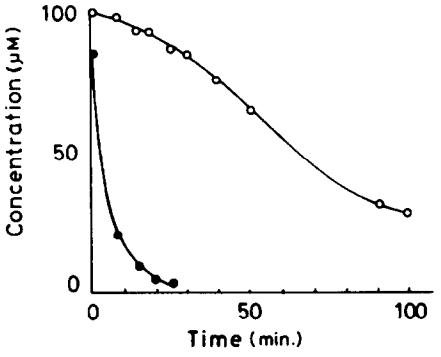
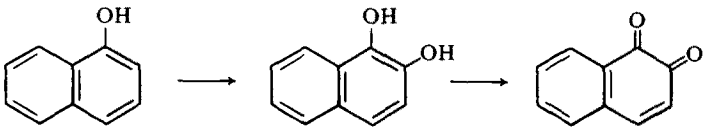


Fig. 5. Non-enzymic interaction of 3,4-anisylquinone and 1-naphthol. 3,4-Anisylquinone (100 μM) was incubated in 0.1 M phosphate buffer pH 7.4 at room temperature in the presence (●——●) or absence (○——○) of 1-naphthol (100 μM). The disappearance of 3,4-anisyslquinone was measured by HPLC as described in Materials and Methods.

of the quinone (Fig. 4a). That these differences reflected changes in the activity of the enzyme was apparent from considering the rate of removal of the substrate. In the absence of 1-naphthol, the rate of removal of 4-hydroxyanisole was approximately exponential (Fig. 4b). In the presence of 1-naphthol, the disappearance of 4-hydroxyanisole was greatly reduced after 2 min (Fig. 4b). However, these results also indicated an initial stimulation of tyrosinase activity, in the presence of 1-naphthol, since there was a rapid removal of about 60% of the substrate in 2 min compared to about 15% in the control incubation (Fig. 4b). The failure to exhibit a corresponding peak value of orthoquinone production (Fig. 4a) was shown to be due to a direct interaction between 1-naphthol and 3,4-anisylquinone which resulted in the rapid removal of the quinone from the reaction mixture. The kinetics of this reaction are illustrated in Fig. 5. In these experiments the rate of removal of synthetic 3,4-anisylquinone from a buffer solution was monitored by HPLC and compared with the rate of its removal when 1-naphthol was present in equimolar amounts. Similar experiments with 4-hydroxyanisole showed that there was no significant direct reaction between the two substrates.

DISCUSSION

[1^{14}C]-1-Naphthol is metabolized by tyrosinase to naphthoquinones (Fig. 1) and covalent binding species (Fig. 2). In the presence of NADPH, the major methanol-soluble metabolite observed at early times (Fig. 1a and 1b) co-chromatographed with 1,2-naphthoquinone. At later times the radioactivity, which co-chromatographed with 1,2-naphthoquinone, decreased whereas radioactivity co-chromatographing with 1,4-naphthoquinone increased (Fig. 1c). By analogy with the tyrosinase oxidation pathway [14] formation of 1,2-naphthoquinone (**III**) by tyrosinase would occur by *o*-hydroxylation of 1-naphthol (**I**) and oxidation of 1,2-dihydroxynaphthalene (**II**):



I II III

Like other quinones, naphthoquinones are highly reactive molecules and may bind covalently to cellular macromolecules either directly, or possibly as their corresponding naphthosemiquinones. The observation that in the absence of a reducing agent (NADPH) essentially all the [1-^{14}C]-1-naphthol (500 μM) was covalently bound to tyrosinase within 10 min, whereas in the presence of NADPH the covalent binding occurred at later times (Fig. 2) suggested that reduction of the quinone to the corresponding hydroquinone reduced its reactivity.

We have previously shown that ethylenediamine reacts fairly specifically with 1,2- but not with 1,4-naphthoquinone [9]. The metabolic studies (Fig. 1) together with the finding that ethylenediamine inhibited the tyrosinase-mediated binding of 1-naphthol suggested that much of the covalent binding was due to 1,2-naphthoquinone or a metabolite derived from it. The inability of ethylenediamine to completely inhibit the covalent binding due to 1-naphthol suggested that other species, as well as 1,2-naphthoquinone, were involved in the binding or that the ethylenediamine had limited access to the site of generation and/or binding of the quinone.

1,2-Naphthoquinone shortened the lag period for 4-hydroxyanisole oxidation. This was in marked contrast to the considerable lengthening of the induction period which occurred when the enzyme had been pre-exposed to 1-naphthol (Fig. 3). The difference in the response of the enzyme when the quinone was present externally compared to its generation as an oxidation product suggests that the enzyme alteration has to be in the vicinity of the active site in order to inhibit its activity.

Initially a marked activation of tyrosinase was observed as measured by an increased rate of removal of 4-hydroxyanisole during the first 2 min (Fig. 4b). We have previously observed a similar stimulatory action on tyrosinase by rhodazonic acid, which may act as an electron acceptor without gaining access to the active site of the enzyme [15]. This brief period of stimulation was followed by a decreased rate of 4-hydroxyanisole removal (Fig. 4b). Inactivation of tyrosinase by its oxidative activity is a well-established phenomenon in several different systems [16–18]. There is evidence that binding of phenolic oxidation products to tyrosinase occurs [19], and this is given further support by our results with 1-naphthol.

The effect of pre-incubation with 1-naphthol on the kinetics of mushroom tyrosinase is complicated both by the extent to which inhibition of the oxidation of the alternative monophenolic substrate, 4-hydroxyanisole, is due to substrate competition, and also by the interaction of 1-naphthol with 3,4-anisylquinone, the first oxidation product of 4-hydroxyanisole oxidation (Fig. 5). Thus, while 1,2-naphthoquinone, or some derivative of it, binds strongly to the enzyme some of the apparent inactivation of the enzyme was not due to this direct effect. Although it is not possible to be certain of the mechanism of action, our evidence strongly favours the proposal that the major inactivation of mushroom tyrosinase results from binding of an oxidation product of 1-naphthol.

The metabolic activation of 1-naphthol by tyrosinase to reactive metabolites, such as 1,2-naphthoquinone and covalently bound species suggests that 1-naphthol or a structurally related compound may be of potential therapeutic benefit in the treatment of melanomas which are high in tyrosinase activity. It remains to be demonstrated that mammalian tyrosinase possesses the same range of substrate utilization as the mushroom enzyme. In preliminary experiments we have shown that 1-naphthol is cytotoxic to both melanotic and amelanotic B-16 melanoma cultures, but no apparent selectivity was observed.

In summary, we have shown that 1-naphthol is metabolized by mushroom tyrosinase to 1,2-naphthoquinone and covalently bound products, which are largely derived from naphthoquinone. This covalent binding modified the kinetics of tyrosinase-catalysed oxidation of 4-hydroxyanisole, a well characterized substrate of tyrosinase. These results raise the possibility that 1-naphthol or a related derivative might be of therapeutic potential in the treatment of melanomas.

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