Mechanism of Selective Incorporation of the Melanoma Seeker 2-Thiouracil into Growing Melanin

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The mechanism of selective incorporation of 2-thiouracil (TU), a highly specific melanoma seeker, into growing melanins was investigated both in vitro and in vivo. Methods used included direct analysis of the melanins, by evaluation of the absorption at 350 nm (A_{350}) and chemical degradation coupled with HPLC quantitation of pigment markers, i.e., pyrrole-2,3-dicarboxylic acid (PDCA) and pyrrole-2,3,5-tricarboxylic acid (PTCA), as well as biosynthetic experiments involving tyrosinase-catalyzed oxidation of DOPA, 5,6-dihydroxyindole (DHI), and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Injection of radiolabeled TU into melanoma-bearing mice resulted in a rapid incorporation of the drug into the tumor pigment, with a substantial decrease in A_{350} and in PTCA yields. Similar changes in the absorption properties were observed in biosynthetic melanins prepared in the presence of TU, whereas the yields of PTCA and PDCA varied depending on the pigment precursor used. When incubated with DOPA in the presence of tyrosinase, TU profoundly modified the normal course of melanogenesis, favoring formation of a complex mixture of addition products consisting mainly of 6-*S*-thiouracil-DOPA as well as DHI−TU adducts. The latter were obtained in larger amounts by enzymatic oxidation of DHI in the presence of TU and were identified as the 3- and 2-substituted adducts **1** and **2**, the dimer **3**, and the trimer **4**. Similar reactions carried out on DHICA yielded the 4-substituted adduct **5**, the dimer **6**, and the trimer **7**. A new mechanistic scheme for the incorporation of TU into growing melanin is proposed, which envisages nucleophilic attack of the thioureylene moiety of TU to transient quinonoid intermediates in the melanin pathway, chiefly dopaquinone and 5,6-indolequinones, followed by entrainment of the resulting adducts into the growing pigment *via* oxidative copolymerization with DHICA and/or DHI.

Introduction

The dramatic rise in incidence and mortality of cutaneous melanoma, a highly aggressive tumor which selectively affects the melanocytes, and the exceedingly low rate of response to immunotherapy and radiotherapy as well as conventional chemotherapeutic modalities have urged, over the past decade, the quest for innovative treatment strategies aimed at effectively targeting disseminated tumor while sparing normal tissues.[1] One approach which is actively being pursued in various laboratories[2] centers on the possibility of localizing tumor metastases and delivering radionuclides or cytotoxic moieties by means of specific tumor seekers directed toward the peculiar metabolic pathway of melanocytes leading to melanin. A schematic outline of melanogenesis in melanocytes is presented in Scheme 1. In the early stages, oxidation of tyrosine by the copper enzyme tyrosinase leads to dopaquinone, a labile quinone which rapidly cyclizes to give dopachrome, *via* leucodopachrome. The latter aminochrome, which is the first UV detectable intermediate of melanogenesis, may undergo rearrangement with and/or without concomitant decarboxylation to give 5,6-dihydroxyindole (DHI) and/or 5,6-dihydroxyindole-2-carboxylic acid (DHICA), respectively.[3] Both the latter step and the subsequent oxidative polymerization processes occur within highly specialized organelles termed melanosomes and are coarsely or finely modulated by a variety of enzymes and regulatory factors which may affect the pigment pathway at different levels and with different modalities.[4]

Among the various molecules currently under scrutiny as potential melanoma seekers, the antithyroid drug 2-thiouracil (TU) and related thioureylene compounds hold special promise because of their peculiar ability to selectively accumulate into *de novo* synthesized melanin in overactive melanin-producing systems without binding to preformed pigment.[5] Thus, in spite of ontoward effects due to some thyroidal accumulation, TU and thioureylene compounds provide a unique means to localize the therapeutic agent into the target cells. Incorporation of TU was initially demonstrated by autoradiography in embryonic and fetal eyes[6] where melanin synthesis takes place at high rate, but subsequent experiments revealed a selective uptake in melanotic melanoma transplanted to mice[7] as well as in murine melanoma metastases[7c,8] and cultured human melanoma cells.[9] TU tagged with radioiodine for clinical application in melanoma scanning exhibited selective localization in melanoma tissues[8,10] and gave promising results in pilot studies on patients with ocular melanoma[11a] or disseminated cutaneous melanoma.[11b] Boronated TU derivatives have also been investigated for application in boron neutron capture therapy.[12]

In spite of the potential of TU for early diagnosis and treatment of disseminated melanoma, progress toward the development of new TU-related substances and their practical exploitation in the clinics has been considerably slowed down by the lack of detailed knowledge

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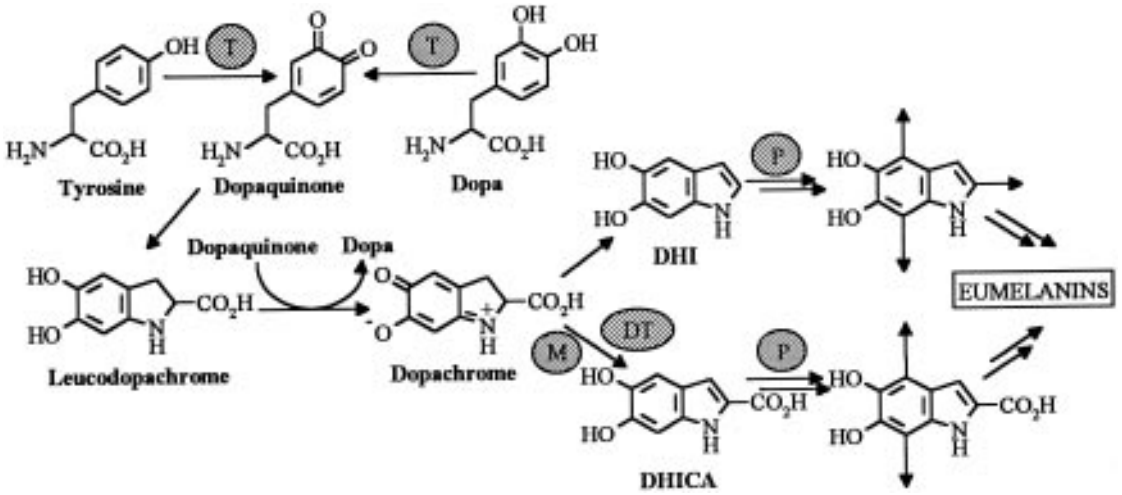
Scheme 1. Schematic Outline of Melanin Biosynthesis from Tyrosine Highlighting the Role of Tyrosinase and Other Enzymatic and Nonenzymatic Regulatory Factors[a]

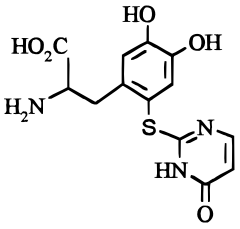


[a] T = tyrosinase; P = peroxidase; M = metal ions; DT = dopachrome tautomerase.

about the chemical events underlying selective incorporation into active melanin-producing systems.

Early investigations by Whittaker[13] showed that incorporation of TU into the pigment fraction of melanin-synthesizing cells was proportional to tyrosinase activity and associated with a marked alteration of the phenotypic properties of the resulting pigment. On this basis, it was suggested that TU could directly react with some quinonoid intermediates in the melanin pathway, giving rise to covalent adducts which were subsequently incorporated into the growing pigment polymer. In line with this view, Larsson and colleagues[14] demonstrated that an *S*-benzyl derivative of TU or sulfur-lacking analogues failed to incorporate into melanin-producing tissues, underlining the critical role of the free thioureylene moiety of TU in the process.

The first direct insight into the mechanism of incorporation of TU into melanin was gained some years ago in an in vitro study[15] showing that TU is able to bring nucleophilic attack to enzymatically produced dopaquinone to give an addition product identified as 6-*S*-thiouracil-DOPA (TU−DOPA). The importance of this reaction for the binding of TU to growing melanins was confirmed in a subsequent work;[16] however, to what extent the trapping of dopaquinone could account for the overall effects of TU on melanogenesis in vivo remained an open issue.



TU-Dopa

We report herein the results of a detailed study aimed at addressing the mechanism of action of TU by an integrated approach involving direct chemical analysis of melanins from TU-treated melanoma tumors compared to synthetic pigments and an in-depth investigation of the effect of TU on the various stages of the biosynthesis of melanins in vitro. The new data which emerge from this study expand the previous mechanistic framework to include 5,6-dihydroxyindoles as new critical targets of TU in the melanin pathway.

Table 1. Incorporation of [2-^{14}C]TU and Analysis of Melanins from Murine B16 Melanoma Tumors

time of assay (min)	specific incorporation (cpm/mg of melanin)	A_{350}[a]	PTCA (ng/mg)[b]
0	nd	0.071 ± 0.005	1125 ± 62
30	2500	0.032 ± 0.002	493 ± 31
90	2600	0.036 ± 0.003	560 ± 38
180	2300	0.043 ± 0.004	589 ± 40

[a] Determined on 0.1 mg/mL solutions, average ± SD (n = 3). [b] Average ± SD (n = 3).

Results

Incorporation of TU into Melanoma Melanin. Initial experiments were directed at chemically characterizing the pigments from murine melanoma tumors after treament of the mice with [2-^{14}C]TU. Melanins were isolated from the tumors at different times after intraperitoneal injection of the radioactive drug and analyzed by a standard procedure,[17] involving oxidation of the pigment with alkaline hydrogen peroxide and HPLC quantitation of resulting degradation products, namely, pyrrole-2,3,5-tricarboxylic acid (PTCA) and pyrrole-2,3-dicarboxylic acid (PDCA). The former arises by oxidative disruption of DHICA or 2-substituted DHI units, whereas the latter is a typical marker of DHI units with a free 2-position.[17] The absorbance at 350 nm of a solution of melanosomes obtained by treatment in hot alkali was taken as a measure of the pigment content of the tissue.[18]

Data in Table 1 show that most of the incorporation of [2-^{14}C]TU occurred within 30 min after inoculation. Although difficulties in the isolation and purification of the pigment from the tissue prevented an accurate determination of the actual pigment content,[19] a well-apparent decrease in the A_{350} following administration of TU was observed. Notably, formation of PTCA by chemical degradation was also found to decrease after injection of TU with respect to the control pigment. Whether these changes reflect an inhibitory effect of TU on pigment synthesis within the melanosomes or an alteration of melanin structure and intrinsic chro-

Table 2. Spectrophotometric and Chemical Analysis of Synthetic Melanins

sample	TU/ substrate molar ratio	melanin yield[a] (w/w)	A_{350}[b]	PTCA yield (ng/mg)	PDCA yield (ng/mg)
DOPA melanin		42	0.87	3600	1100
TU−DOPA melanin	0.25	50	0.61	4000	4600
	0.50	43	0.55	4600	7600
DHI melanin		94	0.93	5246	4780
TU−DHI melanin	0.50	136	0.50	1809	7280
DHICA melanin		90	0.58	58000	
TU−DHICA melanin	0.50	62	0.42	48000	

[a] Average of two determinations. [b] Determined on 0.1 mg/mL solutions.

mophoric properties consequent to covalent binding of the drug could not be deduced from these data and required separate analysis of the effect of TU on pigment formation in vitro.

Preparation and Properties of TU-Containing Synthetic Melanins. To gain more direct insight into the effect of TU on the structure and chemical properties of melanins, various TU-containing melanins were prepared by tyrosinase-catalyzed oxidation of DOPA, DHI, and DHICA in the presence of different amounts of TU. DOPA was preferably used in place of tyrosine because of more favorable kinetic parameters for the enzymatic oxidation, all other features and chemical course being virtually identical. Table 2 reports the A_{350} as well as the yields of PTCA and PDCA of TU-containing melanins in comparison with control pigments prepared in the absence of TU. TU/substrate molar ratios not greater than 0.5 were preferably chosen to permit formation of sufficient amounts of pigment for chemical characterization. In all cases TU caused a consistent decrease of the A_{350} values which may be ascribed to an increase in the molecular weight of the pigment polymer due to incorporation of TU and/or to structural modifications affecting the absorption properties.

Markedly different trends were observed in the yields of PTCA and PDCA, depending on the nature of the pigment precursor. In particular, PTCA decreased with TU incorporation in DHI and DHICA melanins but increased in DOPA melanins, whereas PDCA increased both in DOPA and DHI melanins. The yields of formation of the melanins were also different, the highest values being obtained for DHI−TU melanins; by contrast TU incorporation produced an apparent lowering of the pigment yields in the case of DHICA melanins and no substantial changes in those of DOPA melanins which were invariably rather low.

Effect of TU on the Tyrosinase-Catalyzed Oxidation of Tyrosine. The effect of TU on melanogenesis in vitro was investigated by periodical HPLC monitoring of the course of the tyrosinase-catalyzed oxidation of DOPA in the presence and absence of TU (Figure 1). In the presence of 2:1 molar excess of TU with respect to DOPA, the reaction course was markedly affected leading to, besides some DHI (peak II), a complex pattern of products (lower panel).

The major of these, I, was identified as TU−DOPA by comparison of the chromatographic and spectrophotometric properties with those of an authentic sample obtained by oxidation of tyrosine in the presence of TU, as described previously.[15] The other components (peaks III−VIII) could derive either from oxidation of TU−

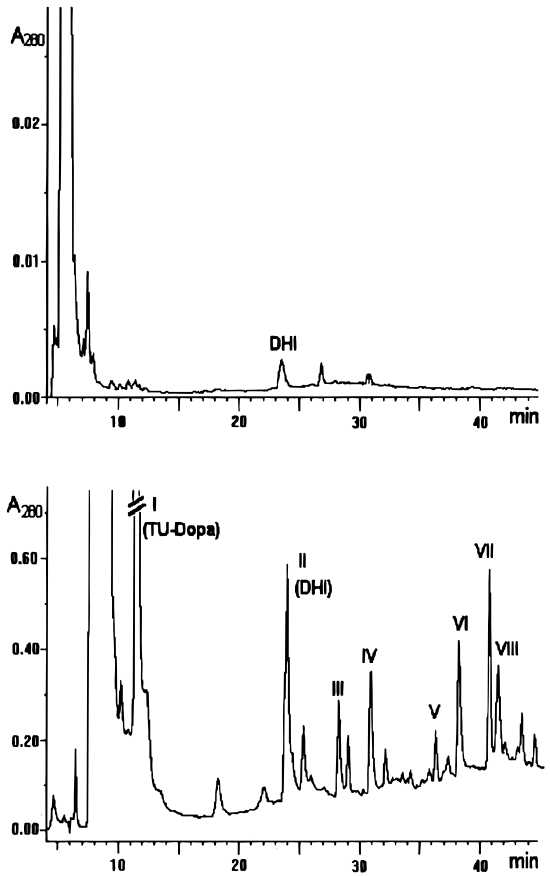


Figure 1. HPLC elution profile of the products formed by oxidation of DOPA (1.5 mM) by tyrosinase (70 U/mL) in the absence (upper panel) and presence (lower panel) of TU (3 mM) in 0.1 M phosphate buffer, pH 7.0, at 70 min reaction time. Eluant system II was used for analysis; all other conditions were as described in the Experimental Section.

DOPA or from reaction of TU with melanin intermediates distal to dopaquinone. The former possibility was apparently ruled out by separate experiments on the enzymatic oxidation of TU−DOPA with tyrosinase, which showed the initial formation of a yellow chromophore (λ_{max} 450 nm) gradually turning brown without giving any distinct peak coeluting with peaks III−VIII on HPLC analysis.

The origin of products eluting under peaks III−VIII was thus addressed by investigating the effect of TU on the rearrangement of dopachrome. Addition of the drug at various concentrations to a solution of pure dopachrome generated by chemical oxidation of DOPA with silver oxide under anaerobic conditions did not modify the rate of spontaneous conversion of the aminochrome to 5,6-dihydroxyindoles, as evidenced by both spectrophotometrically monitoring the decay of the absorption maximum at 475 nm and HPLC analysis, showing the presence of DHI as the main product. Conversely, TU profoundly affected the oxidative polymerization of 5,6-dihydroxyindoles, as evidenced by analysis of the HPLC elution patterns as well as from comparison of the absorption properties of the developing pigment polymers in the presence and absence of TU. Based on these results, the focus was then shifted toward the effect of TU on the oxidation of indolic melanin precursors catalyzed by tyrosinase.

Effect of TU on the Oxidative Polymerization of DHI. Aerial oxidation of DHI catalyzed by tyrosinase

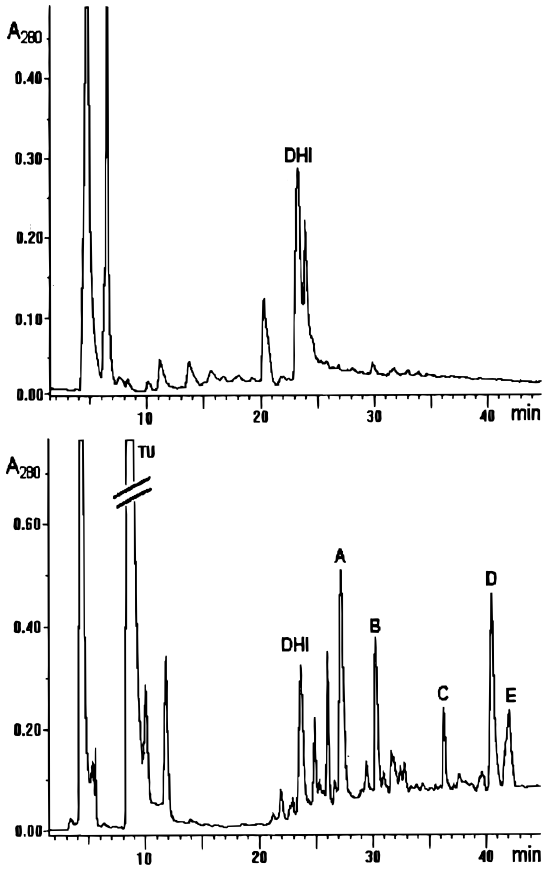


Figure 2. HPLC analysis of the mixtures obtained by oxidation of DHI (1.5 mM) by tyrosinase (70 U/mL) in the absence (upper panel) or presence (lower panel) of TU (3 mM) in 0.1 M phosphate buffer, pH 7.0, at 40 min reaction time. Eluant system II was used for analysis; all other conditions were as described in the Experimental Section.

was performed at pH 7.0 with substrate concentrations in the range $10^{-5}-10^{-3}$ M and in the presence of varying molar ratios of TU. HPLC analysis of the reaction mixtures in the early stages showed a gradual alteration of the elution profiles with increasing concentrations of the substrates and/or TU/DHI molar ratios. With 1×10^{-3} M DHI and 2 mol equiv of TU, the usual pattern of oligomerization of the indole was virtually suppressed, being replaced by a new set of main products labeled as A−E in the elutogram in Figure 2 (lower panel).

Appropriate scale up of the experimental protocol and optimization of the HPLC elution conditions for preparative purposes allowed isolation of the major reaction products in sufficient amounts for structural analysis. The two less retained peaks, A and B, exhibited virtually identical MALDI mass spectra with a pseudomolecular ion peak at *m/z* 279 indicating isomeric 1:1 DHI−TU adducts. The proton spectrum of compound A exhibited, besides a pair of doublets at δ 5.93 and 7.60 (J = 6.4 Hz) due to the H-6 and H-5 protons of the pyrimidine ring of TU, two 1H singlets at δ 6.70 and 6.82 and a broad 1H singlet at δ 7.35, indicating a C-3-substituted DHI in which the H-2 proton resonated markedly downfield. Analysis of the ^{13}C NMR spectrum showed CH carbons at δ 97.92, 102.71, and 130.37 for the C-7, C-4, and C-2 carbons, in that order, as well as a resonance at δ 94.31 for the C-3 quaternary carbon. Overall, these data indicated for the product the structure of 5,6-dihydroxy-3-[(4-hydroxypyrimidin-2-yl)thio]-indole (**1**).

Formulation of compound B as the regioisomer **2** followed from analysis of the pattern of resonances in the proton spectrum compared to that of **1**. Particularly indicative was the lack of the signal due to the H-2 proton, replaced by a broad doublet at δ 6.40 (J = 1.6 Hz) arising from a deshielded H-3 proton. The resonances of the C-3 and C-2 carbons at δ 109.55 and 118.31, respectively, were in accord with the above conclusion. As an aside, it is worth noting that in both **1** and **2** TU substitution caused a significant shielding of the ipso position and an opposite effect on the adjacent position in the ^{13}C NMR spectrum of DHI.[20]

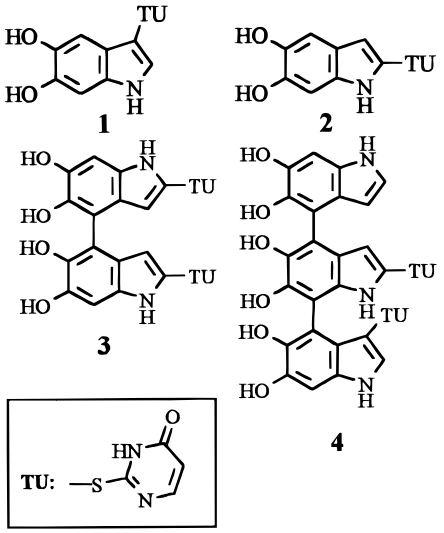
The pyrimidine ring in the adducts **1** and **2** may exist in various tautomeric forms in which the mobile hydrogen is borne by the oxygen or one of the two nitrogen atoms. Consideration of the values of the carbon resonances of the adducts in comparison with those of the free TU system and of the upfield shift of the C-2 carbon resonance would point to a pyrimidin-3-one structure; discrimination between the remaining tautomers is more difficult, although the marked changes of the proton and carbon resonances at the 6-position may be taken as evidence of localization of a C−N double bond on the adjacent position.

The spectral data of the minor component of the mixture eluting under peak C provided evidence for a symmetric dimer of a 1:1 DHI−TU adduct. The mode of linking of the indole units and the position of attachment of the TU unit followed from analysis of the carbon spectrum in which the pattern of resonances observed for the adduct **2** could be easily recognized, with the noticeable exception of the lack of the C-4 CH resonance, which was replaced by a deshielded quaternary carbon at δ 109.99, indicating that such a position was engaged in a C−C bond. Accordingly, compound C was formulated as 2,2′-bis[(4-hydroxypyrimidin-2-yl)-thio]-5,5′,6,6′-tetrahydroxy-4,4′-biindolyl (**3**).

A most significant feature of the HPLC elutogram was the presence of the highly retained peaks D and E eluting at about 41 and 42 min. The collected peaks were analyzed and fractionated using different elutographic systems: While peak D was found to consist of a major component eluting invariably as a single band, peak E showed in most cases a complex mixture of TU containing DHI oligomers (MALDI-MS and NMR evidence) which could not be further purified.

The compound eluted at 41 min was a trimer of DHI containing two TU units, as evidenced by the presence of pseudomolecular peaks at *m/z* 696 $(M + H)^+$ and 718 $(M + Na)^+$ in the MALDI-MS spectrum. Inspection of the proton spectrum, aided by homonuclear decoupling experiments, provided evidence for the two TU moieties (overlapped pairs of doublets at δ 6.02 and 7.63) and a C-4-substituted DHI unit (two 1H double doublets at δ 6.16 and 7.00 for the H-3 and H-2 protons and a 1H broad singlet at δ 6.81 for the H-7 proton with a long range coupling with the 6.16 signal). A 1H singlet at δ 6.70 for a H-7 proton, a broad singlet at δ 6.92, and a doublet (J = 2.5 Hz) at δ 7.32 were also present, the latter two ascribable to H-2 and H-3 protons on different indole rings. These assignments were supported by 2-D carbon−proton shift correlation experiments, which

showed direct coupling between the protons at δ 6.70 and 6.81 and two C-7 carbon resonances at δ 97.22 and 97.46, respectively, between the δ 6.16 proton and a C-3 carbon at δ 100.68, and between the δ 7.00 proton and a C-2 carbon at δ 123.78. The remaining two proton resonances at δ 6.92 and 7.32 correlated with carbons at δ 103.73 and 128.46, respectively. These data, coupled with those of the adducts **1** and **2**, allowed identification of the remaining units of the trimer as a 2-TU-substituted, 4- and 7-linked and a 3-TU-substituted, 4-linked 5,6-dihydroxyindole. Assemblage of these units is however not univocal, as it may occur in two distinct modes differing in the position of attachment of the outer units to the C-4 and C-7 positions of the central 2-TU-substituted unit. Attempts to discriminate between these structures by long range C−H correlation experiments proved inconclusive because of the difficulty to detect significant C−H interring couplings. However a weak but distinct NOE effect could be observed between the H-3 protons at δ 6.16 and 6.92. On this basis, the compound was eventually assigned structure **4**, in which two 3-unsubstituted indole units are opposed because of the linkage through the 4-position.



Overall isolated yield of products **1**−**4** was about 20%. This was lower than the mass balance from HPLC analysis (50%) and should be ascribed to material losses during the rather delicate purification process. In this connection, it should be emphasized that the direct isolation of 5,6-dihydroxyindoles from oxidation mixtures is an arduous task because of their pronounced oxidizability in aqueous media and the tendency to polymerize to insoluble intractable materials. Noticeably, formation of products **1**−**4** is still significant at DHI concentrations much lower than 10^{-3} M, e.g., 1×10^{-5} M, in the presence of 2 mol equiv of TU, though the efficiency of the trapping diminishes consistently.

Effect of TU on the Oxidative Polymerization of DHICA. The ability of TU to affect the oxidative conversion of DHICA to melanin pigments was investigated at various concentrations under conditions similar to those described in the case of DHI. Complete diversion of enzymatic conversion of DHICA to oligomer intermediates (chiefly the 4,4′- and 4,7′-biindolyls)[21] was observed with 1×10^{-3} M DHICA and 0.5×10^{-3} M TU, i.e., at a lower TU/indole molar ratio compared to

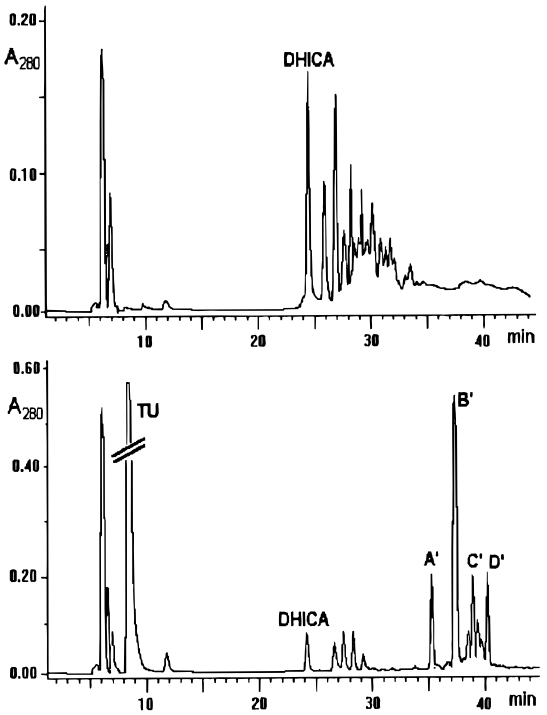


Figure 3. HPLC analysis of the mixtures obtained by oxidation of DHICA (1.5 mM) by tyrosinase (70 U/mL) in the absence (upper panel) or presence (lower panel) of TU (3 mM) in 0.1 M phosphate buffer, pH 7.0, at 40 min reaction time. Eluant system I was used for analysis; all other conditions were as described in the Experimental Section.

DHI. This is reasonably due to the higher stability of the transient quinonoid species generated by oxidation of DHICA.

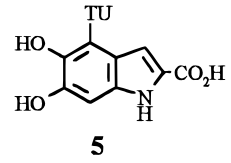
A typical HPLC profile of the mixture obtained by oxidation of 1×10^{-3} M DHICA in the presence of 2×10^{-3} M TU is shown in Figure 3 (lower panel). The elutogram consisted, besides some starting indole, of four peaks (A′−D′), none of which corresponded to known DHICA oligomers.

The component eluting after 35 min (peak A′) could be isolated by preparative HPLC. This was assigned structure **5** mostly by analysis of the MALDI-MS spectrum, exhibiting a pseudomolecular ion peak at *m*/*z* 320 for a DHICA−TU adduct, and of the ^{13}C NMR spectrum which showed typical CH resonances for C-3 and C-7 carbons. Notably, the substituted C-4 carbon resonated at δ 121.02, i.e., significantly downfield with respect to that of the parent indole,[21] whereas the adjacent C-5 carbon experienced a shielding effect. This indicates that substitution by TU causes opposite effects on the ipso and adjacent positions of DHICA compared to those observed in the case of DHI.

The most abundant product eluting under peak B′proved on MALDI analysis to be a dimer containing one TU moiety. Scrutiny of the carbon resonance pattern revealed two CH signals at δ 97.14 and 97.92, which correlated with two singlets in the proton spectrum at δ 6.78 and 6.82, suggesting two unsubstituted C-7 carbons, and a CH resonance at δ 107.50 for a C-3 carbon, correlating with a broad 1H singlet at δ 6.37. Overall, these data allowed formulation of the compound as the dimer **6**. This structural assignment was substantiated by long range C−H shift correlation experiments. In particular, the H-3′ proton (δ 6.37) showed ^3J coupling with the C-4′ carbon (δ 107.00) and ^2J

coupling with the C-8′ carbon (δ 135.51); the H-7 proton (δ 6.78) exhibited ^3J coupling with the C-9 (δ 122.38) and C-5 (δ 135.34) carbons and ^2J or ^4J couplings with the C-8 (δ 131.66), C-6 (δ 145.67), and C-2 (δ 125.90) carbons; the H-7′ proton (δ 6.82) showed ^3J coupling with the C-3′ CH (δ 107.12), C-9′ (δ 120.73), and C-5′ (δ 143.26) carbons and ^2J couplings with the the C-8′ (δ 135.51) and C-6′ (δ 147.86) carbons. Moreover $^3J/^2J$ couplings were observed between the NH and NH′ protons (δ 11.15 and 11.34) and the C-9/C-9′ (δ 122.38, 120.73) and C-2/C-2′ (δ 125.90, 127.13) carbons.

Repeated fractionation of the peaks eluting at around 38−40 min afforded very minute amounts of a product which exhibited a pseudomolecular ion peak at *m/z* 702, indicating a trimer bearing one TU unit. Unfortunately, attempts to obtain a complete spectral characterization of the compound, including the ^{13}C NMR spectrum, were hampered by its exceedingly low yield coupled with a marked instability to autoxidation. However, close inspection of the ^{1}H NMR and DEPT spectra revealed four unsubstituted ring positions, namely, one C-3, one C-4, and two C-7 positions. The H-3 proton resonance (δ 7.51) was markedly deshielded with respect to the corresponding values in **5** and **6** (δ 6.52 and 6.37, respectively), suggesting the lack of a spatially proximate TU unit on the 4-position, as in the latter. On these grounds, and partly on the basis of chemical considerations, the trimer was tentatively assigned structure **7**.



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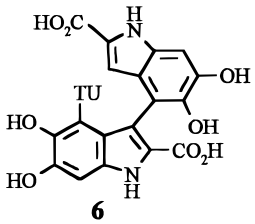

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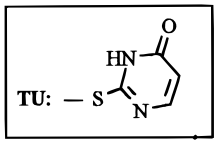

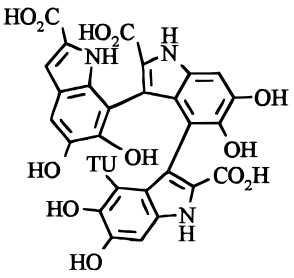

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Relevance of Indole−TU Conjugates to the Mechanism of Incorporation of TU into Growing Melanins. The observed facility of DHI and DHICA to undergo oxidative conjugation with TU prompted us to address the role of these reactions in the mechanism of incorporation of TU into melanin in vitro.

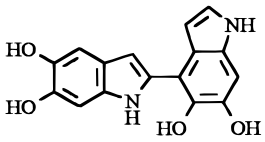
As a first relevant observation, it was found that adducts **1**−**4**, derived from DHI, correspond in fact to the main components of the mixture obtained by tyrosinase-catalyzed oxidation of tyrosine in the presence of TU, marked as peaks III−V and VII in Figure 1. The failure to detect DHICA−TU conjugates can be explained considering that under the normal conditions of the tyrosinase-catalyzed oxidation of tyrosine in vitro, little or no DHICA is formed by rearrangement of dopachrome, as the reaction proceeds mainly with concomitant decarboxylation.[22]

Table 3. Comparative Analysis of TU−Indole Adducts and DHI or DHICA Oligomers

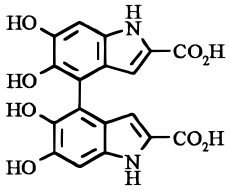
compd	PTCA yield (molar %)	PDCA yield (molar %)
8	1.5	6
3		
9	17	
6	5	

On enzymatic oxidation with tyrosinase, adducts **1**, **2**, and **5** smoothly decomposed to afford grayish materials bearing little or no resemblance to the dark pigments formed from the parent indoles under similar conditions. Spectrophotometric monitoring showed, in all cases examined, a progressive flattening of the indole absorption maximum in the UV region and the concomitant rise of a featureless absorption in the range 400−600 nm, without significant scattering due to insoluble melanin-like pigments.

With a view to assessing the influence of TU units on the oxidative breakdown of the indole rings to PTCA and PDCA, TU-containing oligomers **3** and **6** were subjected to chemical degradation in comparison with representative DHI and DHICA dimers, i.e., **8**[17,23] and **9**.[21] The data in Table 3 indicate a drop in the molar yield of PTCA passing from the DHICA dimer **9** to the TU-containing dimer **6**. The TU−DHI dimer **3** did not give rise to any detectable PTCA, whereas dimer **8** containing a 2-substituted indole unit gave the pyrrole in significant yield. No PDCA was formed from **3**.



8



9

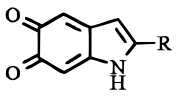
Discussion

Uptake of TU in melanoma and other melanin-producing tissues is generally agreed to involve free passage of the drug into melanocyte cytoplasm followed by selective accumulation into melanogenic compartments where tyrosinase activity is sufficiently high.[7b] The crucial gap in the above sequence of events, however, was at what level(s) and by what mechanism TU affects the biosynthetic pathway of melanogenesis and is eventually retained into growing pigment. From the bulk of evidence emerging from the present and previous studies,[15,16] it is now possible to conclude that selective incorporation of TU into newly synthesized melanin hinges on a chemical interaction process targeted to reactive quinonoid intermediates which are transiently generated in the melanogenic compartment. The major outcome of the present study is, in particular, the demonstration that, besides dopaquinone, TU may also affect diffusible intermediary products formed in the later stages of melanogenesis. This finding expands considerably the range of options offered to TU for incorporation into overactive melanocytes and includes those intracellular sites, typically the melanosomes, where 5,6-dihydroxyindoles may reach locally high concentrations and be exposed to an oxidizing environment.[24]

Chemically, the mechanism of interaction of TU with the melanogenic pathway bears some resemblance to

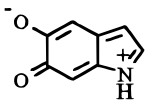
that established for sulfhydryl compounds, such as cysteine and glutathione. These latter compounds can react with both dopaquinone, to give cysteinyl- and glutationyl-DOPA adducts,[3,22] and subsequent intermediates, such as dopachrome[25] and 5,6-dihydroxyindoles,[26] to give addition products. A major difference, however, lies in the regioselectivity of the conjugation reactions. While SH compounds, like cysteine and glutathione, react with dopaquinone to give mainly 5-*S*-cysteinyl-DOPA, with small amounts of the 2-*S* and 6-*S* adducts,[27] TU, which exists largely in the thione form, adds almost exclusively at the 6-position of dopaquinone.[28]

Coupling with DHI has been shown to occur at both the 2- and 3-positions, which would point to a significant electron deficiency at those sites in a short lived quinonoid intermediate. This, however, is difficult to rationalize in terms of structures like **10**, commonly reported for 5,6-indolequinones.[29] A plausible explanation could envisage either a significant contribution of structure **11** to the resonance hybrid or a tautomerization step leading from **10a** to the quinone methide **12**, which would expectedly be more proclive to react at C-3. Conversely, reaction of TU with the oxidation product of DHICA proved to be regioselective, involving conceivably nucleophilic attack of TU to the C-4 site of the *o*-indolequinone **10b**.

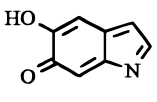


10a: R=H

10b: R=CO_2H



11



12

Another critical point for discussion is the mode of polymerization of TU−indole adducts. Chain elongation in the case of TU−DHI adducts proceeds through the 4- and 7-positions, without significant involvement of the 2-position, as commonly observed in aqueous buffer.[23] This can be ascribed to the steric hindrance opposed by the TU moiety against the approaching indole unit, though electronic effects should also be considered. More or less similar reasonings may be used to account for the regiochemical features of the polymerization of DHICA−TU adducts. The structures of **6** and **7** denote a mode of coupling of the indole via the 3- and 4- or 7-positions. This represents a minor route of the oxidative polymerization of DHICA which may become prevailing under certain conditions, as recently pointed out.[21]

The observed differences in color and solubility properties between the melanins prepared in the presence of TU and the control pigments obtained in the absence of the drug are evidently the result of the incorporation of relatively bulky TU moieties into the growing pigment. This affects the process of melanogenesis not only for what concerns the extent and mode of elongation of the pigment polymer, as evidenced in the case of the TU−indole adducts, but also with regard to the oxidizability and intrinsic absorption properties of the TU-containing units. The reduced yield of formation of PTCA from degradation of TU−DHI melanin, being determined on a weight-by-weight basis, is undoubtedly expected to reflect the contribution of non-indolic TU units to the molecular weight of the resultant polymer but should also be ascribed to the presence of a large number of 2- or 3-TU-substituted indole units, which cannot give rise to PTCA. The significantly lower A_{350} value of TU−DHI melanin with respect to the reference melanin is also in line with this interpretation.

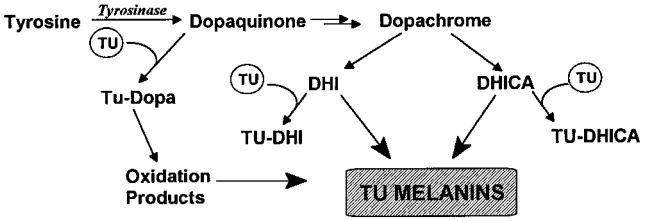
Taking the yields of PDCA as being roughly proportional to the number of terminal DHI units (unsubstituted at C-2), it can likewise be reasoned that the higher PDCA/PTCA ratio of the TU-containing DHI melanin compared to the control pigment is an index of a lower degree of polymerization in the former.[17] In the case of DHICA melanins, the variation in the yield of PTCA is more difficult to interpret and may possibly be ascribed to the different solubility properties of the TU-containing vs control melanin, whereby the fraction which precipitates from the oxidation mixture, and is subjected to analysis, may not entirely reflect the bulk of the TU-containing oligomers, which remain preferentially in solution. Consistent with this view is the lower yield of formation of TU−DHICA melanin with respect to DHICA melanin and the modest difference in the A_{350} values.

Similar arguments may be applied to rationalize the data pertaining to TU-containing DOPA melanins. Here, TU−DOPA is a major intermediate in the process of pigment formation. However, its inability to cyclize to produce indolic melanin precursors, the actual source of pyrrole fragmentation products, would entail that it contributes to a minor extent to the buildup of the pigment polymer and that a large proportion of the melanin, i.e., the pigmented insoluble fraction of the reaction mixture, derives from interaction of TU with 5,6-dihydroxyindole intermediates. Consistent with this interpretation, the observed increase in the yields of PTCA as well as in those of PDCA with increasing TU concentration would indicate firstly that the isolated TU-containing pigment is richer in indolic units compared to the standard DOPA melanin, in which part of the units do not cyclize, and secondly that it resembles more closely a TU−DHI melanin. This is well in accord with the known tendency of dopachrome to undergo rearrangement under the usual conditions of melanogenesis in vitro to give mainly DHI.[22]

Although the extension *tout-court* of the in vitro data to the in vivo situation is critical because of the difficulty to obtain pure melanoma melanin and the lack of suitable techniques for direct structural analysis of melanins, it appears from the data in Table 1 that the TU-affected tumor pigment bears a gross resemblance to DHICA melanin for what concerns PTCA formation, the changes in A_{350}, and the failure to give PDCA. This is well consistent with the recent demonstration of the central role of DHICA in the biosynthesis of melanoma melanin[30] and would indirectly support a central role of TU−DHI interactions in the process of incorporation of TU into melanoma tissues.

In conclusion, in the light of the present results and those from previous studies, it is possible to propose an improved mechanism for incorporation of TU into growing melanin. This is schematically outlined in Scheme 2 and involves reaction of TU with quinonoid melanin precursors formed at various stages of the biosynthetic pathway, to give 1:1 adducts. Though poorly melanogenic *per se*, these latter are susceptible of oxidation and

Scheme 2. Overview of the Proposed Mechanism of Incorporation of TU into Growing Melanins Showing the Main Sites of Intervention of the Drug in the Biosynthetic Pathway



may partake in the polymerization process to different extents and with different modalities. The resulting pigment would consist of an intimate mixture of TU-containing structures akin to those obtained by oxidation of DHI and DHICA, which exhibit overall a lower degree of polymerization, different solubility, and altered light-absorption properties compared to the parent melanin.

Experimental Section

UV spectra were performed with a Perkin-Elmer Lambda 7 spectrophotometer having the cell compartment controlled at 25 ± 0.1 °C with circulating water. MALDI mass spectra were taken on a Reflex time-of-flight spectrometer, operating in positive linear mode; ions, formed by a pulsed UV laser beam (nitrogen laser, $\lambda = 337$ nm), were accelerated at 15 keV, matrix: 2,5-dihydroxybenzoic acid. ^{1}H (^{13}C) NMR spectra were recorded at 400.1 (100.6) or 270.1 (67.9) MHz on Bruker WM 400 and AC 270 spectrometers. Tetramethylsilane was used as reference standard. 2-D carbon−proton shift correlation experiments were carried out at 100.6 MHz using a Bruker XHCORR microprogram with a D_3 delay corresponding to J values of 140 and 10 Hz. Experiments were performed with 128 × 2048 matrix sizes. HPLC was carried out on a Gilson apparatus equipped with a 305 model pump and a 316 UV detector.

Materials. 2-Thiouracil, L-DOPA, mushroom tyrosinase (E.C. 1.14.18.1, *o*-diphenol:O_2 oxidoreductase, 3900 units/mg), and horseradish peroxidase (donor:H_2O_2 oxidoreductase, E.C. 1.11.1.7) type II (220 units/mg, RZ $E_{430}/E_{275} = 2.0$) were from Sigma Chemicals (St. Louis, MO). Soluene 350 was from Packard (Downers Grove, IL). DHI and DHICA were synthesized according to a standard procedure.[31] [2-^{14}C]Thiouracil (specific radioactivity 17.57 mCi/mmol) was obtained from Izinta Isotope Trading Enterprise (Budapest, Hungary). PTCA and PDCA were prepared as reported.[17] Dimers **8**[17] and **9**[21] were prepared as previously described. B16 melanoma cells were obtained and passaged in mice as reported.[30]

Analytical Conditions. Analytical HPLC was performed using a Spherisorb S5 ODS2 column (4.6 × 250 mm) or an Econosil C18 10 μm (10 × 250 mm) column for analytical or preparative runs, respectively. The flow rate was maintained at 1 or 6 mL/min. Detection was set at 280 nm. Different isocratic or gradient elution conditions were employed as follows: 0.05 M citrate, pH 2.5, containing 5% CH_3CN (solvent A), CH_3CN (solvent B), 0−5 min 100% solvent A, 5−50 min from 0% to 35% (eluant system I) or from 0% to 50% (eluant system II) solvent B; 0.05 M triethylammonium formate, pH 4 (solvent A), CH_3CN (solvent B), 0−10 min 10% solvent B, 10−40 min from 10% to 40% solvent B, 40−50 min 40% solvent B (eluant system III); 1% AcOH (solvent A), CH_3CN (solvent B), 0−15 min 100% solvent A, 15−45 min from 0% to 40% (eluant system IV) or from 0% to 25% (eluant system V) solvent B; 0.1 M phosphate buffer, pH 2.1−MeOH, 95:5 (eluant VI).

Reaction of TU with Melanin Precursors. In a typical experiment a solution of DOPA, DHI, or DHICA (1.5 mM) in 0.1 M phosphate buffer, pH 7.0, was treated with tyrosinase (70 U/mL) and a solution of TU (3 mM) under vigorous stirring at room temperature. When DOPA was used as substrate, the TU solution was added dropwise during the first 15 min of the reaction. Aliquots of the reaction mixtures were periodically withdrawn, treated with solid ascorbic acid (1 mg/mL solution), and analyzed by HPLC using the eluant system II in the experiments with DOPA and DHI or the eluant system I in those run with DHICA. In some experiments dopachrome generated by chemical oxidation of DOPA (0.5 mM) with silver oxide as described[32] was treated with varying amounts of TU (0.5−1.5 mM), and the course of the reaction was followed spectrophotometrically at 475 nm. HPLC analysis of the reaction mixtures at complete disappearance of the red tint was carried out using eluant system II.

Isolation of DHI−TU Adducts 1−4. A solution of DHI (225 mg, 1.5 mmol) and TU (385 mg, 3 mmol) in 0.1 M phosphate buffer, pH 7.0 (1 L), was treated with tyrosinase (94 U/mL) under stirring. After 50 min, the reaction was stopped by addition of solid ascorbic acid (500 mg) and the mixture extracted repeatedly with ethyl acetate (5 × 200 mL). The combined organic layers were dried over sodium sulfate and evaporated to dryness. The residue (250 mg) was taken up in DMSO and fractionated by preparative HPLC using the eluant system III to afford five main bands with t_R 8, 13, 18, 22, and 24 min. The first of these was further purified using the eluant system IV to afford, beside some DHI, compound **1** in pure form (20 mg, 5% yield). Removal of the saline components of the t_R 13 and 22 bands was achieved under reverse phase conditions using the eluant system IV and yielded compounds **2** (15 mg, 4% yield) and **4** (30 mg, 9% yield), respectively. The band at t_R 19 min was rechromatographed with the eluant system V as the mobile phase to give compound **3** (8 mg, 2% yield). The slowest moving band proved a complex mixture of products under most analytical conditions and was not further fractionated. Formation yields of compounds **1**−**4** were estimated in the HPLC elutograms of the reaction mixtures by integration of the areas of the peak corresponding to **1** vs calibration curves. Purity of compounds **1**−**4** was estimated ≥95% by inspection of NMR spectra and HPLC analysis using eluant systems II and III as mobile phase.

5,6-Dihydroxy-3-[(4-hydroxypyrimidin-2-yl)thio]indole (1): UV λ_{max} 298, 274 nm; MALDI-MS *m/z* 276 $(M + H)^+$, 298 $(M + Na)^+$; ^{1}H NMR (DMSO-d_6) δ (ppm) 5.93 (1H, d, J = 6.4 Hz), 6.70 (1H, s), 6.82 (1H, s), 7.35 (1H, bs), 7.60 (1H, d, J = 6.4 Hz), 11.10 (1H, bs); ^{13}C NMR (DMSO-d_6) δ (ppm) 94.31 (C), 97.92 (CH), 102.71 (CH), 109.08 (CH), 121.85 (C), 130.37 (CH), 130.59 (C), 141.66 (C), 143.51 (C), 153.33 (CH), 165.32 (C), 172.32 (C).

5,6-Dihydroxy-2-[(4-hydroxypyrimidin-2-yl)thio]indole (2): UV λ_{max} 307, 285 nm; MALDI-MS *m/z* 276 $(M + H)^+$, 298 $(M + Na)^+$; ^{1}H NMR (DMSO-d_6) δ (ppm) 5.88 (1H, d, J = 6.3 Hz), 6.40 (1H, bd, J = 1.6 Hz), 6.71 (1H, bs), 6.80 (1H, s), 7.64 (1H, d, J = 6.3 Hz), 10.95 (1H, bs, J = 1.6 Hz); ^{13}C NMR (DMSO-d_6) δ (ppm) 96.73 (CH), 104.13 (CH), 108.44 (CH), 109.55 (CH), 118.31 (C), 120.56 (C), 132.83 (C), 140.87 (C), 143.91 (C), 154.01 (CH), 168.05 (C), 172.14 (C).

5,5′,6,6′-Tetrahydroxy-2,2′-bis[(4-hydroxypyrimidin-2-yl)thio]-4,4′-biindolyl (3): UV λ_{max} 302, 277 (infl) nm; MALDI-MS *m/z* 549 $(M + H)^+$; ^{1}H NMR (DMSO-d_6) δ (ppm) 6.30 (1H × 2, d, J = 6.3 Hz), 6.94 (1H × 2, bs), 7.69 (1H × 2, bs), 7.93 (1H × 2, d, J = 6.3 Hz), 8.72 (1H × 2, bs), 8.91 (1H × 2, bs), 11.20 (1H × 2, bs); ^{13}C NMR (DMSO-d_6) δ (ppm) 98.13 (CH), 106.56.06 (CH), 106.84 (CH), 109.99 (C), 118.05 (s, C-2), 119.33 (s, C-9), 133.56 (C), 141.03 (C), 143.93 (C), 151.19 (CH), 162.58 (C), 171.14 (C).

5,5′,5″,6,6′,6″-Hexahydroxy-2′,3-bis[(4-hydroxypyrimidin-2-yl)thio]-4,7′:4′,4″-terindolyl (4): UV λ_{max} 302, 274 (infl) nm; MALDI-MS *m/z* 696 $(M + H)^+$, 718 $(M + Na)^+$; ^{1}H NMR (DMSO-d_6) δ (ppm) 6.02 (2H, d, J = 6.5 Hz), 6.16 (1H, dd, J = 2.7, 2.5 Hz), 6.70 (1H, s), 6.81 (1H, bs), 6.92 (1H, bs′), 7.00 (1H, dd, J = 2.7, 2.6 Hz), 7.32 (1H, d, J = 2.5 Hz), 7.63 (2H, d, J = 6.5 Hz), 8.20 (1H, bs), 8.62 (2H, bs), 9.21 (3H, bs), 10.74, 10.75, 10.76 (1H each, bs); ^{13}C NMR (DMSO-d_6) δ (ppm) 97.22 (CH), 97.34 (C), 97.46 (CH), 100.55 (C), 100.63 (CH), 103.71 (CH), 105.00 (C), 108.09 (2 × C), 108.24 (2 × CH), 120.42 (C), 121.76 (C), 121.97 (C), 122.12 (C), 123.67 (CH), 128.34 (CH), 130.16 (C), 133.59 (C), 134.22 (C), 140.96 (2 × C), 143.08 (C),

143.14 (C), 144.99 (2 × C), 153.48 (CH), 153.75 (CH), 158.66 (C), 158.75 (C), 172.21 (2 × C).

Isolation of DHICA−TU Adducts 5−7. Oxidation of DHICA (290 mg, 1.5 mmol) in the presence of TU (385 mg, 3 mmol) in 0.1 M phosphate buffer, pH 7.0 (1 L), promoted by tyrosinase (56 U/mL) was carried out essentially as described in the case of the preparative scale reaction of DHI. After 2 h, the reaction was stopped by addition of solid ascorbic acid (500 mg), and the mixture was acidified to pH 3 with phosphoric acid and worked up as above. The residue was purified by preparative HPLC using the eluant system I to afford four bands at t_R 28, 30, 34, and 36 min. The t_R 28 band was found to consist of the starting indole. Removal of the saline components of the t_R 30 and 34 bands using the eluant system IV yielded compounds **6** (45 mg, 12% yield) and **5** (25 mg, 10% yield), respectively. Repeated chromatography of the t_R 36 band using eluant system III, followed by desalting as above, afforded compound **7** (2 mg, 0.5% yield). Purity of compounds **5**−**7** (≥95%) was evaluated by inspection of NMR spectra and HPLC analysis using elution conditions I and III.

2-Carboxy-5,6-dihydroxy-4-[(4-hydroxypyrimidin-2-yl)thio]indole (5): UV λ_{max} 333, 275 (infl), 262 nm; MALDI-MS *m/z* 320 $(M + H)^+$; ^{1}H NMR (DMSO-d_6) δ (ppm) 5.90 (1H, d, J = 6.3 Hz), 6.52 (1H, bs), 6.94 (1H, s), 7.45 (1H, d, J = 6.3 Hz), 11.44 (1H, bs); ^{13}C NMR (DMSO-d_6) δ (ppm) 100.15 (CH), 107.07 (CH), 107.94 (CH), 121.02 (C), 122.39 (C), 128.08 (C), 135.12 (C), 136.79 (C), 147.57 (C), 153.97 (CH), 158.57 (C), 162.30 (C), 171.04 (C).

2,2′-Dicarboxy-5,5′,6,6′-tetrahydroxy-4-[(4-hydroxypyrimidin-2-yl)thio]-3,4′-biindolyl (6): UV λ_{max} 340 (s), 324, 255 nm; MALDI-MS *m/z* 511 $(M + H)^+$, 533 $(M + Na)^+$; ^{1}H NMR (DMSO-d_6) δ (ppm) 6.04 (2H, d, J = 6.3 Hz), 6.37 (1H, bs), 6.78 (1H, s), 6.82 (1H, bs′), 7.65 (1H, d, J = 6.3 Hz), 11.15 (1H, bs), 11.34 (1H, bs); ^{13}C NMR (DMSO-d_6) δ (ppm) 97.14 (CH), 97.92 (CH), 107.00 (C), 107.12 (CH), 107.50 (CH), 108.53 (CH), 120.73 (C), 121.64 (C), 122.38 (C), 125.90 (C), 127.13 (C), 131.66 (C), 135.34 (C), 135.51 (C), 143.26 (C), 145.67 (C), 147.86 (C), 154.17 (CH), 158.71 (C), 162.44 (C), 162.62 (C), 171.41 (C).

2,2′,2″-Tricarboxy-5,5′,5″,6,6′,6″-hexahydroxy-4-[(4-hydroxypyrimidin-2-yl)thio]-3,4′:3′,7″-terindolyl (7): UV λ_{max} 340 (s), 327, 255 nm; MALDI-MS *m/z* 702 $(M + H)^+$; ^{1}H NMR (DMSO-d_6) δ (ppm) 6.11 (1H, d, J = 6.4 Hz), 7.02, 7.03 (1H each), 7.22 (1H, s), 7.46 (1H, d, J = 6.4 Hz), 7.51 (1H, bs); DEPT (DMSO-d_6) δ (ppm) 99.43 (CH), 100.37 (CH), 105.55 (CH), 105.79 (CH), 154.20 (CH).

Preparation of Melanins. Melanins were obtained by tyrosinase-catalyzed oxidation of DOPA, DHI, and DHICA in the presence or absence of TU according to a modification of procedures previously reported.[17] In brief, a solution of the appropriate substrate (1.0 mmol) in 0.1 M phosphate buffer, pH 7.0 (670 mL), was treated with tyrosinase (1240 units) under a stream of oxygen in a thermostatic bath at 25 °C. When necessary, a solution of TU was added dropwise soon after the enzyme over a period of about 15 min up to the desired concentration. After 30 min a second aliquot of the enzyme (1240 units) was added, and the incubation mixture was taken under oxygen stream for 4 h. The pigment formed was precipitated by acidification to pH 4, collected by centrifugation after storing at 5 °C overnight, and washed extensively with water. All melanins were dried over silica gel and sodium hydroxide overnight and equilibrated with saturated calcium chloride.

Analysis of Oligomers, Melanins, and Melanosomal Tissues. Degradation of melanins by treatment with alkaline hydrogen peroxide under controlled atmosphere was performed by a modification of the reported procedures.[17,33] A suspension of the appropriate oligomer or melanin (2 mg) or melanosomal tissue from melanoma (4 mg) in 1 M NaOH (1 mL/mg of sample) was treated with 30% H_2O_2 (30 μL/mg of sample) and vigorously stirred at 25 °C for 3 h. The reaction was stopped by addition of 5% $NaHSO_3$ (50 μL/mL), and the mixture was acidified to around pH 2 with 1 M HCl and extracted with ethyl acetate (4 × 1 mL). The combined organic layers were dried over sodium sulfate and evaporated to dryness. The residue was taken up in water (1 mL), and the yields of PTCA and PDCA were determined by HPLC using eluant VI as the mobile phase. The yields reported are the average of three separate experiments, with SD not exceeding ±7%.

Spectrophotometric determination of melanin content was performed by the method of Ito et al.[18] A suspension of the tissues or melanins in 1 M NaOH (1 mg/mL) was treated with 1% hydrogen peroxide and taken at 80 °C in a water bath. After 45 min the absorbance at 350 nm of the resulting solution was recorded. The A_{350} values reported are the means of three determinations, with SD ≤ ±10%.

Incorporation Experiments. Pathogen free female C57Bl/6 mice (6 weeks old) were challenged subcutaneously with B16 melanoma cells as previously described.[30] Two weeks after challenge, the mice were treated intravenously with 10 μCi of [2-^{14}C]TU. At times noted in Table 1, the animals were quickly euthanized by CO_2 inhalation and the tumors dissected free of surrounding tissue, weighed, and then rapidly frozen in a dry ice/ethanol bath. After homogenization of the tumors in 1% acetic acid−ethanol (30:70, v/v) and centrifugation,[30] melanosomes were purified by sucrose density gradient ultracentrifugation as described.[16] Melanins were dried and taken to constant weight as detailed above. Weighed aliquots of the labeled melanins were dissolved in Soluene 350 and counted in a liquid scintillation counter.

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