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Synthesis and Preliminary in Vitro Biological Evaluation of 4-[(4-Hydroxyphenyl)sulfanyl]but-3-en-2-one, a 4-Mercaptophenol Derivative Designed As a Novel Bifunctional Antimelanoma Agent

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We report the synthesis and preliminary in vitro biological evaluations of 4-[(4-hydroxyphenyl)sulfanyl]but-3-en-2-one, a compound designed as a potential bifunctional antimelanoma agent, bearing both a tyrosinase-activatable phenolic moiety and a GSH-reactive α,β-unsaturated carbonyl group. Both the *E* (**1**) and *Z* (**2**) isomers of the synthesized compound proved to be very good substrates of mushroom tyrosinase, reacted quickly with GSH at physiological pH, and showed a significant cytotoxic activity against B16F1 murine melanoma cells.

Introduction

Malignant melanoma is an extremely aggressive tumor of melanocytic origin. Like normal melanocytes, melanoma cells synthesize melanin pigments through a complex pathway in which a crucial role is played by tyrosinase (EC 1.14.18.1). This enzyme catalyzes both the rate-limiting oxidation of L-tyrosine to L-DOPA and the subsequent conversion of L-DOPA to L-DOPA quinone, a highly reactive *o*-quinone yielding melanins through a series of enzymatic and nonenzymatic reactions.[1] Because tyrosinase is frequently upregulated in melanoma cells, phenolic and catecholic compounds have been investigated as tyrosinase-activated antimelanoma prodrugs, ultimately producing alkylating cytotoxic *o*-quinones. Examples are 4-hydroxyanisole (**4-HA**[a], Figure 1), 4-*S*-cysteaminylphenol, and 4-*S*-cysteaminylcatechol, all having shown antimelanoma activity both in in vitro and in in vivo studies.[2−5] Because a byproduct of melanogenesis is hydrogen peroxide, which can easily cross cell membranes and that, if not appropriately processed, can generate cytotoxic reactive oxygen species, GSH and cytosolic GSTs (EC 2.5.1.18), are critically important to the maintenance of cells of melanocytic origin. Melanomas, as well as other tumors, often exhibit upregulation of both GSH and some GSTs, in particular GSTP1-1,[6] these events likely contributing to tumor resistance to some anticancer agents.[7] In this regard, a novel therapeutic strategy based on GST-activated anticancer prodrugs has been recently proposed.[8−11]

In this work, we report the synthesis of a novel bifunctional antimelanoma agent, 4-[(4-hydroxyphenyl)sulfanyl]but-3-en-2-one, obtained as *E* and *Z* isomers (**1** and **2**, respectively, Figure 1), characterized by the simultaneous presence of a tyrosinase-activatable phenolic moiety and a GSH-reactive α,β-unsaturated carbonyl group, this latter conferring reactivity toward GSH.[9] Three further compounds with different substitutions at the 4-mercapto group and lacking the α,β-unsatured moiety (**3**−**5**, Figure 1) were also synthesized for the purpose of comparison. All the synthesized compounds were tested in vitro for their rate of oxidation by mushroom tyrosinase, their spontaneous and GST-catalyzed reaction with GSH, and their cytotoxic activity against B16F1 murine melanoma cells.

Results and Discussion

Tyrosinase/O_2-Mediated Oxidation of 1−5 and 4-HA. The time-course of tyrosinase/O_2-mediated oxidation of **1**−**5** and **4-HA**, a prototypical tyrosinase-oxidizable phenol,[2] was analyzed by a UV−vis spectroscopy method. In the absence of tyrosinase, all compounds showed a maximum absorbance peak in the 250−320 nm range (see Figure 2 in Supporting Information). Upon the addition of tyrosinase to **1**−**4** and **4-HA**, the absorbance of the respective maximum absorbance peak decreased and a characteristic peak appeared at 460 nm, thus indicating the formation of an *o*-quinone.[12] Surprisingly, **5** did not exhibit these modifications of its UV−vis spectrum, suggesting that methylation of the carboxyl group prevents oxidation of **4** by tyrosinase.

Kinetic analyses were carried out by measuring the initial rate of tyrosinase-catalyzed *o*-quinone formation in the presence of increasing concentrations of **1**−**4** and **4-HA**. Compounds **1**−**4** exhibited similar affinity for the enzyme, their K_m values (ranging from 20 to 36 μM) being lower than that of **4-HA** (about 47 μM). By contrast, significant differences were found in V_{max} and k_{cat} values of the compounds, the rank order of these parameters being **2** > **1** ≫ **3** > **4** > **4-HA**. Overall, mean kinetic parameters for the oxidation of **1**−**4** and **4-HA** by tyrosinase (summarized in Table 2 in Supporting Information) indicate that **1** and **2** are the best among the herein studied tyrosinase substrates.

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[a] Abbreviations: GSH, glutathione; GST, glutathione S-transferase; **4-HA**, 4-hydroxyanisole.

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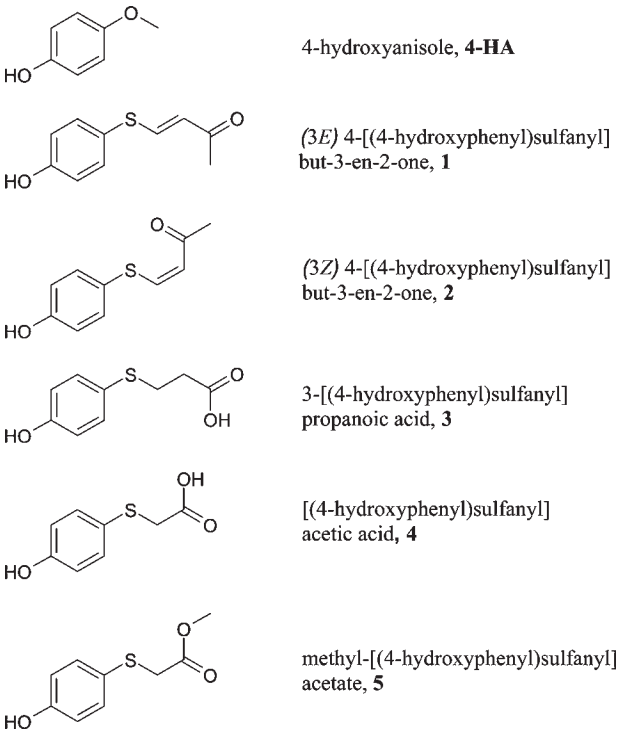


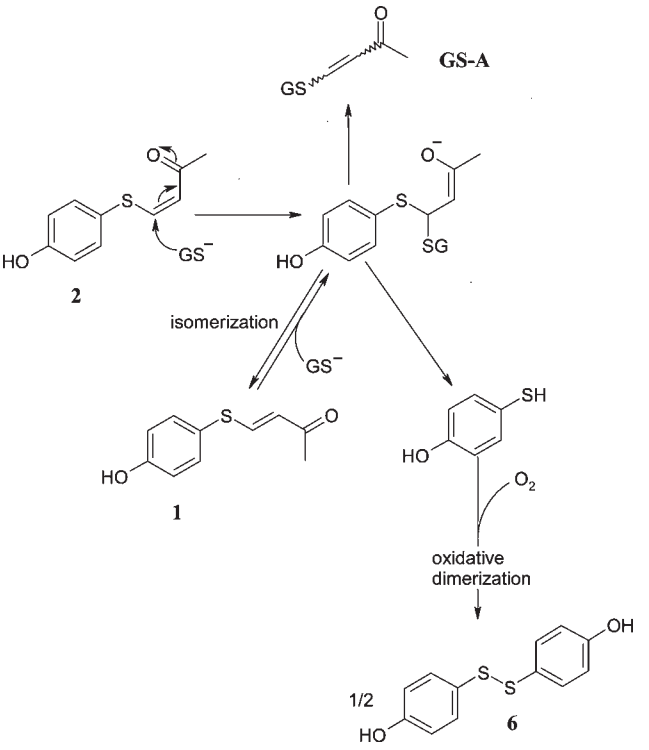
Figure 1. Chemical structures of **4-HA** and compounds **1**−**5**.

Reactivity toward GSH. The rate of the nucleophilic attack of GSH on the β-carbon of α,β-unsaturated carbonyl compounds is known to be pH-dependent.[10] Thus, the reactivity of each synthesized compound toward GSH was evaluated by incubation with GSH for increasing times (0−120 min) at different pH values (6.0, 7.4, and 8.0). Compounds **3**−**5**, which lack the α,β-unsatured carbonyl moiety, did not show any detectable reactivity toward GSH (data not shown), whereas **1** and **2** reacted with GSH in a pH-dependent manner (Figure 3 in Supporting Information). While at pH 6.0 the rate of the reaction of **1** and **2** with GSH was negligible, at pH 7.4 and 8.0, the reaction proceeded at a much higher rate and followed pseudo-first-order kinetics. $t_{1/2}$ values for **1** were 20.5 and 6.1 min at pH 7.4 and 8.0, respectively, and $t_{1/2}$ values for **2** were 38.6 and 18.4 min at pH 7.4 and 8.0, respectively.

The interaction of **1** and **2** with GSH was further investigated at pH 7.4 either in the presence or in the absence of rat liver cytosol, as a source of GSTs. Rat liver cytosol markedly increased the reactivity of both compounds toward GSH (data not shown), and $t_{1/2}$ values of 2.2 and 7.24 min were obtained for **1** and **2**, respectively.

The reaction of **1** or **2** with GSH yielded two major products, which were separated by HPLC (Figure 4 in Supporting Information) and then identified by ESI-MS. A first product was identified as the expected GSH−butenone adduct (**GS-A** in Scheme 1), originating from the addition of GSH to the activated double bond. The second product was the geometrical isomer of the parent compound (Scheme 1), the conversion of the *Z* isomer (**2**) into the *E* isomer (**1**) being more rapid and occurring at a greater extent than the reverse reaction. This reaction is very efficient for the α,β-unsaturated compounds[13] and proceeds via addition−elimination of GSH to the double bond.[14] According to the proposed Scheme 1, **GS-A** formation should occur via elimination of the 4-mercaptophenol moiety.

Scheme 1. Proposed Scheme for the GSH-Dependent Metabolism of Compound **2**



However, in our experimental conditions, this species was never found as a product of the reaction. By contrast, the incubation mixtures of **1** or **2** with GSH and rat liver cytosol contained a new compound with retention time and *m*/*z* value identical to that of 4,4′-dihydroxydiphenyl disulfide (**6**, Scheme 1), the product of spontaneous oxidation of 4-mercaptophenol.

Thereafter, we synthesized **6** and evaluated some of its biochemical properties. Compound **6** was a good substrate for mushroom tyrosinase, being provided with the lowest K_m value (16.44 ± 2.29 μM) among the prepared compounds. Its V_{max} value (0.234 ± 0.012 μM s^{-1}), which was significantly higher than that obtained with **3**, **4**, and **4-HA**, as well as its k_{cat} and k_{cat}/K_m values (1.52 s^{-1} and 32683, respectively), indicated that tyrosinase catalytic efficiency for **6** was quite similar to that for **2**. Compound **6** was even more reactive than **1** and **2** toward GSH, its $t_{1/2}$ value being less than 1.0 min upon incubation at pH 7.4 in the presence of GSH and rat liver cytosol (data not shown).

Cytotoxic Effects of the Novel Compounds. The last set of experiments evaluated the cytotoxic effects of the synthesized compounds and **4-HA** toward murine melanoma B16F1 cells. The results in Table 1 show that **1**, **2**, and **6** were effective cytotoxic agents provided with similar IC_{50} values (17.4, 19.2, and 21.2 μM for **1**, **2**, and **6**, respectively). They were more active and more potent than **4-HA**, **3**, and **4**. Compound **5**, which is not a substrate for tyrosinase and does not react with GSH, was devoid of cytotoxic effects. Moreover, *N*-acetyl-L-cysteine (NAC), a cysteine donor for GSH synthesis,[15] did not influence the effect of **4-HA** on B16F1 cell viability while it significantly decreased the cytotoxicity of **1**, **2**, and **6** (Table 1). By contrast, 1-phenyl-2-thiourea (PTU), an inhibitor of tyrosinase activity,[16] did not alter the cytotoxicity of **1**, **2**, and **6**, while slightly decreased the effect of **4-HA**. These results

Table 1. Cytotoxic Effects of **1**−**6** and **4-HA** on Murine Melanoma B16F1 Cells: Effects of NAC and PTU

	IC_{50} value (μM)	cell viability at 100 μM (% of control)		
		−	+PTU (0.2 mM)	+NAC (5 mM)
4-HA	nd	61.3 ± 7.1	78.7 ± 5.0	60.0 ± 3.5
1	17.4 ± 2.3	1.3 ± 1.2	0.0	41.4 ± 5.2
2	19.2 ± 1.9	3.1 ± 1.3	0.0	43.8 ± 4.5
3	nd	77.4 ± 12.5		
4	nd	69.4 ± 9.3		
5	nd	101.4 ± 3.5		
6	21.2 ± 5.6	0.2 ± 0.1	0.0	22.5 ± 3.0

The results are means ± SE from three experiments carried out in quadruplicate; nd, not determined.

allow to conclude that: (a) the mechanism responsible for the cytotoxic activity of **1**, **2**, and **6** is different from that of 4-**HA**, (b) although the cytotoxicity of **4-HA** could be dependent on its tyrosinase-mediated metabolism, the cytotoxic effects of **1**, **2**, and **6** are mainly due to their high reactivity toward GSH, (c) **6**, which is a secondary product of GSH reaction with **1** or **2**, could play a role in the biological effects of these compound because its IC_{50} value was quite similar to those of the two parent compounds and it was highly reactive toward GSH.

Conclusions

Overall, our results indicate that **1** and **2** are very interesting and promising lead compounds for future development of potential agents targeting tumors containing a high concentration of GSH and/or expressing high levels of GST.

Experimental Section

(*E*)-, (*Z*)-4-[(4-Hydroxyphenyl)sulfanyl]but-3-en-2-one (1, 2). The compounds were synthesized by dissolving commercially available 4-mercaptophenol in dimethylsulfoxide (DMSO) in a test tube at room temperature. Thereafter, approximately 3 equiv of 3-butyn-2-one were added to the solution. The reaction was allowed to proceed for 30 min, and thereafter the reaction mixture was lyophilized and the isomers were separated and purified by preparative RP-HPLC (see Supporting Information). Fractions containing either *Z* or *E* isomer were collected, combined, and lyophilized. Purity of the final products was greater than 96% as determined by HPLC. Under the reaction conditions used, the ratio of *E* to *Z* isomers formed was approximately 1:1.

(*E*)-4-[(4-Hydroxyphenyl)sulfanyl]but-3-en-2-one (1). R_f: 0.60^{I}, 0.68^{II}. ^{1}H NMR (DMSO-d_6): H1 δ 2.12 (3H, s), H3 δ 5.58 (1H, d), H4 δ 7.86 (1H, d), H7,11 δ 7.31 (2H, d), H8,10 δ 6.86 (2H, d), H OH δ 9.77 (1H, s). $[M + H]^+ = 195.04$ m/z, calcd for $C_{10}H_{10}O_2S$ 194.04 Da.

(*Z*)-4-[(4-Hydroxyphenyl)sulfanyl]but-3-en-2-one (2). R_f: 0.650^{I}, 0.70^{II}. ^{1}H NMR (DMSO-d_6): H1 δ 2.16 (3H, s), H3 δ 6.40 (1H, d), H4 δ 7.27 (1H, d), H7,11 δ 7.29 (2H, d), H8,10 δ 6.79 (2H, d), H OH δ 9.77 (1H, s). $[M+H]^+ = 195.06$ m/z, calcd for $C_{10}H_{10}O_2S$ 194.04 Da.

Alkylthiophenols (3, 4, and 5). All alkylthiophenols were prepared starting from 4-mercaptophenol. Briefly, appropriate alkyl bromide (4 mmol) dissolved in dichloromethane (DCM) was added dropwise to a stirred solution of 4-mercaptophenol (4 mmol) and diisopropylethylamine (8 mmol) in DCM (10 mL) under nitrogen. After 3 h, the solvent was evaporated in vacuo and the crude product was purified by means of preparative RP-HPLC. Fractions containing the pure product were collected, combined, and lyophilized. Purity of the final products was greater than 96% as determined by HPLC.

3-[(4-Hydroxyphenyl)sulfanyl]propanoic Acid (3). R_f: 0.45^{I}, 0.55^{II}. ^{1}H NMR (DMSO-d_6): H2 δ 2.40 (2H, t), H3 δ 2.91 (2H, t), H6,10 δ 7.21 (2H, d), H7,9 δ 6.72 (2H, d), H OH δ 9.57 (1H, s). $[M - H]^- = 197.03$ m/z, calcd for $C_9H_{10}O_3S$ 198.04 Da.

[(4-Hydroxyphenyl)sulfanyl]acetic Acid (4). R_f: 0.31^{I}, 0.45^{II}. ^{1}H NMR (DMSO-d_6): H2 δ 3.54 (2H, s), H5,9 δ 7.23 (2H, d), H6,8 δ 6.70 (2H, d), H OH δ 9.55 (1H, s). $[M - H]^- = 183.01$ m/z, calcd for $C_8H_8O_3S$ 184.02 Da.

Methyl [(4-Hydroxyphenyl)sulfanyl]acetate (5). R_f: 0.73^{I}, 0.75^{II}. ^{1}H NMR (DMSO-d_6): H1 δ 3.56 (3H, s), H3 δ 3.62 (2H, s), H6,10 δ 7.23 (2H, d), H7,9 δ 6.71 (2H, d), H OH δ 9.63 (1H, s). $[M - H]^- = 197.03$ m/z, calcd for $C_9H_{10}O_3S$ 198.04 Da.

4,4′-Dihydroxydiphenyl Disulfide (6). The compound was synthesized by dissolving 4-mercaptophenol in 1:1 (v/v) H_2O−acetonitrile in a test tube at room temperature. The reaction was allowed to proceed 12 h at room temperature, after which the reaction mixture was lyophilized and the crude product was purified by recrystallization from ethyl acetate/petroleum ether. HPLC analysis showed that purity of the final product was greater than 96%.

R_f: 0.65^{I}; 0.60^{II}. ^{1}H NMR (DMSO-d_6): H5,5′,3,3′ δ 7.25 (2H, d), H6,6′,2,2′ δ 6.73 (2H, d), H OH δ 9.81 (1H, s). $[M-H]^- = 249.00$ m/z, calcd for $C_{12}H_{10}O_2S_2$ 250.34 Da.

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Supporting Information Available: Full experimental details for TLC, RP-HPLC, mass spectrometry, and NMR; for biochemical assays, and for the in vitro cytotoxicity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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