Agents with Potential Specificity against Melanotic Melanoma

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Agents were designed to exploit the high tyrosinase activity in melanotic melanoma relative to normal tissues. If specific tyrosinase activation of these agents occurred, the production of toxic metabolites in the melanoma cells would produce selective cell kill. Synthesis and antitumor activities of three new amino acids, 1a [β -[(p-hydroxyphenyl)amino]alanine hydrochloride], 1b [N^{δ} -(p-hydroxyphenyl)ornithine hydrochloride], and 1c [N^{δ} -(m-hydroxyphenyl)ornithine dihydrochloride], were described. Compounds 1a and 1b were approximately 2-fold more active against the B-16 melanotic melanoma than the amelanotic melanoma cell line in vitro. Compound 1b was approximately 2-fold more potent than compound 1a against either cell line and was 8-fold more potent than L-glutamic acid γ -(4-hydroxyanilide), a natural product isolated from mushroom. No significant growth inhibitory activity was found for the *m*-hydroxy analogue 1c at 100 μ M, the highest concentration tested. Similarly, compound 1b exhibited better activity against P-388 (ED₅₀ = 9.5 × 10⁻⁶ M) than 1a (ED₅₀ = 3.2 × 10⁻⁵ M) and was about 30-fold more potent than 1c. Against human epidermoid carcinoma of the nasopharynx (KB), these agents showed modest inhibitory activity with ED₅₀ values in the range of 1.2 to 3 × 10⁻⁴ M. No in vivo activity against P-388 and B-16 at doses up to 150–200 mg/kg was observed. The biological results suggest that a nonspecific oxidation rather than a specific tyrosinase activation is involved in the biological action of these new compounds.

High tyrosinase activity and high melanin content constitute two of the most significant biochemical characteristics of malignant melanotic melanoma.^{1,2} Selective chemotherapeutic agents for melanotic melanoma could possibly be obtained if a molecule were designed to exploit the significant biochemical differences between melanotic melanoma and normal tissues. The feasibility of this approach has been shown in recent reports on the biochemical studies and selective antitumor properties of L-glutamic acid γ -(4-hydroxyanilide), a natural product isolated from the mushroom Agaricus bisporus.³⁻⁷ The mechanism of action was proposed to involve enzymatic hydroxylation by tyrosinase to produce L-glutamic acid γ -(3,4-dihydroxyanilide), which was oxidized further to an unknown quinone with maximal UV absorbance at 490 nm. A recent report revealed the structure of this 490 quinone to be 2-hydroxy-4-iminoquinone.⁸ This metabolite interacted with sulfhydryl-dependent enzymes, such as α -DNA polymerase, and inhibited tumor growth. Recently, the structure-activity correlations on a number of structural analogues of this natural product and their in vitro antitumor activity against B-16 melanoma have been described.⁹ Although the reported activity of this natural product against B-16 melanoma is only marginal, more active agents might possibly be achieved by design based on the principle of specific enzymatic activation.

In the present study, the synthesis and antitumor activities of β -[(p-hydroxyphenyl)amino]alanine hydrochloride (1a), N^{δ} -(p-hydroxyphenyl)ornithine hydrochloride (1b), and N^{δ} -(m-hydroxyphenyl)ornithine dihydrochloride (1c) are reported. The initial rationale for the design of these new compounds is as shown on Scheme I. It is visualized that intermediates 3 and 4 and product 5 are potential arylators and could be generated in cells with high tyrosinase activity to enable selective cell kill. Though the antitumor properties of 2-hydroxy-1,4benzoquinone (5) have not been studied, the antiviral and antibacterial activities of this compound are documented.^{10,11} An analogous pathway for the generation of 2hydroxy-4-iminoquinone has been reported¹² recently.

Chemistry. The synthesis of β -[(*p*-hydroxyphenyl)amino]alanine hydrochloride (1a) is shown in Scheme II. Ethyl hippurate (7) was subjected to formylation according to the method of Erlenmeyer and Kreutz¹³ to give ethyl Scheme I



formylhippurate (8). Treatment of 8 with p-(benzyloxy)aniline gave the enamine 11, ethyl α -benzamido- β -

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Scheme II



[p-(benzyloxy)anilino]acrylate, instead of the less stable Schiff's base 10.

The structural assignment of enamine 11 was based on the fact that the NMR spectrum of 11 shows no aliphatic protons other than the ethyl ester and benzylic protons. Instead, two exchangeable protons with resonance at δ 8.75 (br d, J = 5.5 Hz) and 8.19 (br s) were observed. The former proton is more readily exchangeable than the latter. Based on the rate of exchange and the splitting pattern,

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Table I. In Vitro Antitumor Activities

compd	cell lines	$ED_{50}, \mu M$
1a	KB ^a	140
	PS^{b}	32
	$M_2 R^c$	24
	A_2^{-d}	54
1b	KB	120
	\mathbf{PS}	9.5
	$M_{2}R$	13
	A_2	22
1c	KB	300
	PS	300
	M_2R	>100
	A_2	>100
L-glutamic acid γ -(4-hydroxyanilide) ^e	M_2R	97
/ () == 0 = y == = = ()	A_2	120

^a $\overline{\text{KB}}$ = human epidermoid carcinoma of the nasopharynx, ^b PS = P388 lymphocytic leukemia. ^c M₂R = melanotic melanoma. ^d A₂ = amelanotic melanoma. ^e Reference 5.

the doublet (δ 8.75) was assigned as amine NH and the singlet (δ 8.19) was assigned as amide NH. These data favor the enamine structure, 11, as the condensation product of 8 and *p*-(benzyloxy)aniline.

The conjugated double bond in 11 is resistant to both catalytic and chemical reductions. When compound 11 was subjected to catalytic reduction (PtO_2 , Rh/C or Pd/C) using ethanol or acetic acid as solvent, the debenzylated product 12 was formed. Treatment of 11 with $NaBH_4$ in boiling dioxane also failed to produce reduction product 14. Instead, a cyclization product, tentatively assigned as 13 based on NMR (lack of an ethyl moiety) and mass spectral data (M = 370), was formed. When compound 11 was treated with $TiCl_3$,¹⁴ p-(benzyloxy)aniline was the only isolable product. However, the reduction of the double bond in 11 proceeded smoothly under acidic conditions with NaBH₃CN^{15a} to give ethyl α -benzamido- β -[p-(benzyloxy)anilino]propionate (14) in high yield. Acid hydrolysis of 14 under nitrogen gave final product 1a in 25% yield with concomitant formation of p-hydroxyaniline, a byproduct which is possibly derived from a reverse Michael addition of 14 or product 1a. The purification of 1a was best achieved by high-performance liquid chromatography using a reverse-phase column.

The synthesis of 1b and 1c are shown in Scheme III. The key intermediate, γ, γ -dicarbethoxy- γ -phthalimidobutyraldehyde (16), was prepared according to the method of Moe and Warner.^{15b} Two approaches were explored to prepare compound 18. The first involved Schiff's base (17) formation followed by NaBH₄ reduction; the second involved reductive amination of aldehyde 16 and p- or m-(benzyloxy)aniline using NaBH₃CN as the reducing agent. While the treatment of aldehyde 16 with p- or m-(benzyloxy)aniline in benzene in the presence of toluenesulfonic acid failed to give the Schiff's base 17, reductive amination gave the desired amine 18 in moderate yield. Selective hydrolysis of the phthalimido function of 18 to give the corresponding amine 19 was achieved with hydrazine. Saponification of 19, followed by decarboxylation in dilute hydrochloric acid, gave the desired target compounds, 1b and 1c.

Antitumor Studies. Compounds 1a-c were tested in vitro against B-16 melanotic melanoma (M_2R), amelanotic

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Scheme III



melanoma (A_2) , P-388 lymphocytic leukemia (PS), and
human epidermoid carcinoma of the nasopharynx (KB)
(Table I). Compounds 1a and 1b were approximately
2-fold more potent against the melanotic melanoma than
the amelanotic melanoma cell line. Compound 1b was
approximately 2-fold more potent than compound la
against either cell line. No significant growth inhibitory
activity was found for the <i>m</i> -hydroxy analogue 1c at 100
μ M, the highest concentration tested. In the same assay,
the ED ₅₀ of L-glutamic acid γ -(4-hydroxyanilide), the
natural product isolated from Agaricus bisporus, was 97
μ M against melanotic (M ₂ R) and 120 μ M against amela-
notic melanoma. These values are in agreement with re-
ported results. ⁹ Thus, the new agents, 1a and 1b, are 4-
to 8-fold more potent than the natural product. No sig-
nificant selective activity of L-glutamic acid γ -(4-
hydroxyanilide) against either melanotic or amelanotic
melanoma was observed. Similarly, compound 1b exhib-
ited better activity against P-388 (ED ₅₀ = 9.5 μ M) than
1a (ED ₅₀ = 32 μ M), and was about 30-fold more potent
than 1c. Against human epidermoid carcinoma of the
nasopharynx (KB), these agents showed modest inhibitory
activity with ED_{50} values in the range of 1.2 to 3×10^{-4}
M

Although it varied in degree, compound 1b was more active than 1a in all four tumor cell lines studied. Because 1a and 1b differ only in amino acid chain length, lipophilicity may be the major factor contributing to this difference in inhibitory activity. While compounds 1a and 1b are 2-fold more active against melanotic than amelanotic melanoma, the difference in inhibitory activity is probably too small to correspond to the 27-fold difference¹⁶ in tyrosinase activity between melanotic and amelanotic melanoma if specific tyrosinase activation is part of mechanism of action in this series of compounds. This consideration is substantiated by the finding that compounds 1a and 1b showed equal or better activity against in vitro P-388, a cell line with no tyrosinase activity, than against B-16 melanotic melanoma. A nonspecific oxidation of the parent compound 1a or 1b to the corresponding *p*-iminoquinone, which is then hydrolyzed to produce the toxic benzoquinone, may be a more likely mechanism of action. This possibility is further supported by the fact that the meta isomer 1c, which cannot be oxidized to an iminoquinone, had minimal activity against all four tumor cell lines. If this explanation proves to be correct, new agents must be designed so that the nonspecific oxidation

Table II. Antitumor Activity against B-16 Melanoma In Vivo

compd	dose, mg/kg	% T/C
1a	150	101
	100	103
	75	111
1b	150	106
	100	98
	75	122
1c	150	82
	100	87
	75	104
L-glutamic acid γ - (4-hydroxyanilide) ^a	400	124
	200	103
	100	120

^a Reference 5.

does not occur prior to the specific desired activation step. Further, the limited substrate specificity of mammalian tyrosinase which has been documented recently¹⁷ should be taken into consideration in the design of new analogues of this class.

Compounds 1a-c were also tested against B-16 melanoma in vivo according to the published National Cancer Institute protocols¹⁸ and were found to be inactive at doses up to 150 mg/kg on the qd 1-9 schedule (Table II). Although L-glutamic acid γ -(4-hydroxyanilide) was reported³ to possess marginal activity against B-16 melanoma, it showed no statistically significant $(T/C \ge 125\%)$ activity at doses up to 400 mg/kg in this study.

Experimental Section

All melting points were taken on Thomas-Hoover capillary melting point apparatus and are uncorrected. Analyses were performed by Galbraith Laboratories, Knoxville, TN. Spectral data were obtained using Perkin-Elmer Infrared Model 727 B and Varian T-60 spectrometers. The NMR data are expressed as δ values relative to tetramethylsilane in CDCl₃ or 3-(trimethylsilyl)propionic acid sodium salt in D₂O. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements are within $\pm 0.4\%$ of the theoretical values. Low-resolution electron-impact mass spectra were obtained on a DuPont 21-492B gas chromatograph-mass spectrometer (GC/MS) system interfaced to a VG 2040 data system. Samples

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were introduced either by direct probe or via a Varian 2740 GC (trimethylsilyl derivatives) coupled to the mass spectrometer by a single-stage glass jet separator. The components of silvlated mixtures were separated on a 1.83 m \times 2 mm i.d. glass column packed with 3% OV-17 on 100-120 mesh Gas Chrom Q and temperature programmed in the range of 160-240 °C. Typical GC operating conditions employed an injector and detector temperature of 245 °C, a 30 mL/min flow rate for both helium carrier gas and hydrogen, and a 300 mL-min flow rate for air. Standard mass spectrometer operating conditions were as follows: jet separator, 210 °C; transfer line, 230 °C; ion source, 260 °C; electron energy, 75 eV; ionizing current, 250 µA; scan speed, 2 s/decade. Derivatization was required before electron-impact mass spectra could be obtained from 1a-c. Microscale silvlation of these compounds was accomplished by solution of 0.5–2.5 mg of each sample in 0.45 mL of a 1:2 mixture of bis(trimethylsilyl)trifluoroacetamide and redistilled acetonitrile in a 3.5-mL glass screw-cap vial equipped with a Teflon-lined rubber septum. The complete silvlation of 1a and 1b could be effected by heating the mixtures at 70 °C for 30 min, while smooth derivatization of 1cwithout rearrangement required reaction at room temperature for several hours. Suitable aliquots $(2-4 \ \mu L)$ of each silvlation mixture were then analyzed by GC or GC/MS.

Ethyl α -Benzamido- β -[p-(benzyloxy)anilino]acrylate (11). Crude aldehyde 8¹³ (28 g, 0.12 mol), p-(benzyloxy)aniline (9; 23.2 g, 0.12 mol), and a catalytic amount of p-TsOH were refluxed in benzene (500 mL) for 3 h. A Dean–Stark water trap was used to remove the water formed during the reaction. Upon cooling, the light yellow crystals were collected, washed with EtOH, and dried to yield 45 g (90%) of the desired product, 11. For elemental analysis, the sample was recrystallized from ethanol: mp 137–142 °C; mass spectrum, m/z (relative intensity) 416 (M⁺, 23), 370 (M – EtOH, 3.5), 325 (46), 279 (12), 105 (100), 91 (52), 77 (34); NMR (CDCl₃) δ 1.32 (3 H, t, CH₃, J = 3.5 Hz), 4.26 (2 H, q, CH₂, J = 3.5 Hz), 5.0 (2 H, s, OCH₂), 6.88 (4 H, s, aromatic), 7.46 (8 H, m, aromatic), 7.82 (3 H, m, aromatic), 8.19 (1 H, s, CONH), 8.75 (1 H, d, J = 5.5 Hz, Ar NH).

Since compound 11 can exist in two isomeric forms, the broad melting point range may indicate that this compound is a mixture of cis and trans isomers. Anal. $(C_{25}H_{24}N_2O_4)$ C, H, N.

Ethyl α -Benzamido- β -(p-hydroxyanilino)acrylate (12). Compound 11 (2 g, 4.8 mmol) and 0.5 g of 10% Pd/C were suspended in 100 mL of EtOH. The mixture was hydrogenated with 35 psi of H₂ overnight. The catalyst was removed by filtration, and the filtrate was evaporated to dryness under reduced pressure. The residue was recrystallized from ethanol and tetrahydrofuran mixed solvent to give white crystals: yield 1.5 g (96%); mp 233-235 °C; mass spectrum, m/z (relative intensity) 326 (M⁺, 57), 280 (M – EtOH, 3.6), 221 (48), 120 (32), 105 (100), 93 (6.7), 77 (39). Anal. (C₁₈H₁₈N₂O₄) C, H, N.

Ethyl α -Benzamido- β -[p-(benzyloxy)anilino]propionate (14). Compound 11 (20 g, 46 mmol) and NaBH₃CN (3 g, 46 mmol) were suspended in 600 mL of absolute methanol. The acidity of the reaction mixture was maintained at pH 4 by the dropwise addition of methanolic HCl. The reaction was monitored by thin-layer chromatography (silica gel; EtOAc-CHCl₃, 1:5, v/v). The time required for the completion of the reaction ranged from 6 to 10 h. The reaction mixture became a clear solution toward the end of the reaction. The solution was evaporated to dryness under reduced pressure. The residue was dissolved in 300 mL of CHCl₃ and filtered, and the filtrate was again evaporated to dryness. The oil was crystallized from aqueous EtOH to give 15 g (75%) of white crystals: mp 127-128 °C; mass spectrum, m/z(relative intensity) 418 (M⁺, 8.5), 327 (26), 212 (54), 105 (100), 91 (47), 77 (34). Anal. (C₂₅H₂₆N₂O₄) C, H, N.

 β -[(p-Hydroxyphenyl)amino]alanine Hydrochloride (1a). Compound 14 (10 g, 23.9 mmol) was refluxed under nitrogen in 100 mL of concentrated HCl for 1.5 h. After cooling, the mixture was extracted with ether twice. The aqueous layer was evaporated to dryness under reduced pressure to give a solid mass, which was purified by C₁₈ reverse-phase column chromatography (55–105 μ m, Waters Associates, Inc.), using H₂O as an eluent. A fraction collector was used to collect 10-mL aliquots, which were monitored by C₁₈ reverse-phase TLC. Fractions with R_f 0.52 (CH₃CN-H₂O, 3:1, v/v) or 0.84 (H₂O) were pooled and lyophilized to give 1.35 g (24%) of the desired product 1a. Recrystallization from ethanol gave white crystals: mp 203 °C (decomposed); ninhydrin test solution or 0.5% KMnO₄ in 1 N NaOH was used to detect the spot on TLC; mass spectrum (as $4Me_3Si$), m/z (relative intensity) 469 (M – CH₃, 0.8), 367 (M – CO₂Me₃Si, 0.8), 291 (62), 266 (100), 219 (11), 193 (56), 178 (60), 147 (27), 101 (31), 73 (95). Anal. (C₉H₁₃N₂O₃Cl) C, H, N, Cl.

 γ,γ -Dicarbethoxy- γ -phthalimidobutyraldehyde (16). An alcoholic solution containing 100 mL of absolute ethanol and 60 mg of sodium was mixed with 20.4 g (67 mmol) of ethyl phthalimidomalonate, and the resulting reaction mixture was cooled to 5 °C with an ice bath. Acrolein (5 mL) was added dropwise with stirring. After the addition of the acrolein was completed, the ice bath was removed and the mixture was stirred for additional 30 min. The mixture was neutralized by the addition of 0.5 mL of glacial acetic acid and filtered. Tht filtrate was evaporated to dryness under reduced pressure and purified with a silica gel column, using CHCl₃-EtOAc (5:1, v/v) as eluent. The front band (R_f 0.68) was collected, pooled, and evaporated to dryness to give a pungent yellowish oil, 2,4-DNP: yield 13 g (54%); mp 164-165 °C (lit. 167-168 °C).^{15b}

 $N-(\gamma,\gamma-\text{Dicarbethoxy-}\gamma-\text{phthalimidobutyl})-p-(\text{benzyl-oxy})$ aniline (18a). Aldehyde 16 (12.0 g, 0.033 mol), p-(benzyl-oxy)aniline (19.8 g, 0.099 mol), and p-(benzyloxy)aniline hydrochloride (7.8 g, 0.033 mol) were suspended in 250 mL of absolute methanol. To the suspension was added NaBH₃CN (2.0 g, 0.033 mol) in small portions, and the mixture was stirred at room temperature overnight. The precipitates were collected, washed with methanol, and dried to give 10.5 g (58%) of white crystals. Recrystallization from EtOH and ether mixed solvent gave white crystals: mp 102–103 °C; mass spectrum, m/z (relative intensity) 544 (M⁺, 16), 453 (77), 233 (20), 187 (36), 160 (33), 146 (54), 104 (43), 91 (100), 76 (33), 65 (30). Anal. (C₃₁H₃₂N₂O₇) C, H, N.

 $N-(\gamma,\gamma-\text{Distributions})-p-(\text{benzyloxy})$ aniline (19a). Compound 18a (11.2 g, 20.6 mmol) and 85% NH₂NH₂ (1.8 mL) were refluxed in 80% EtOH (170 mL) for 2 h. After cooling, the solution was acidified with concentrated HCl to around pH 4 and refluxed for another 2 h. After the solution cooled, the precipitates were removed by filtration. The filtrate was evaporated to dryness under reduced pressure. The residue was suspended in 150 mL of H₂O and filtered, and the filtrate was basified with NaOH solution. The mixture was extracted three times with ether. The ether extracts were combined, dried over Na_2SO_4 , and evaporated to dryness to give 8 g (93%) of oil, which was used for next reaction without further purification: NMR $(\text{CDCl}_3) \delta 1.17 \ (6 \text{ H}, t, \text{CH}_3, J = 3.5 \text{ Hz}), 1.70 \ (2 \text{ H}, m, \text{CH}_2), 2.00$ $(2 \text{ H}, \text{ m}, \text{CH}_2), 2.48 (3 \text{ H}, \text{ s}, \text{NH}_2, \text{NH}), 3.00 (2 \text{ H}, \text{ t}, \text{CH}_2\text{N}, J =$ 2.5 Hz), 4.10 (4 H, q, OCH₂), 4.85 (2 H, s, OCH₂Ar), 6.36 (2 H, d, aromatic H, J = 4.5 Hz), 6.74 (2 H, d, aromatic H, J = 4.5 Hz). For analysis, a small amount of compound was converted to the dihydrochloride salt, which decomposed on heating with no specific melting point (135-145 °C). Anal. (C₂₃H₃₂N₂O₅Cl₂) C, H, N, Cl.

 N^{δ} -(**p**-Hydroxyphenyl)ornithine Hydrochloride (1b). Amine 19a (11.7 g, 0.028 mol) was refluxed in 300 mL of 0.5 N KOH ethanolic solution for 2 h. After cooling, the potassium salt was collected, washed with cold ethanol, dissolved in 150 mL of H₂O, and acidified with concentrated HCl. The mixture was then refluxed for 2 h and the solvent was evaporated to dryness. The residue was suspended in 300 mL of MeOH and filtered, and the filtrate was evaporated to dryness. The amorphous solid was refluxed in 100 mL of concentrated HCl for 6 h. Upon evaporation to dryness, the residue was recrystallized from EtOH to give 4.5 g (61%) of the final product 1b: mp 257 °C (decomposed); mass spectrum (as 4Me₃Si), *m/z* (relative intensity) 512 (M⁺, 23), 497 (M - CH₃, 0.9), 395 (M - CO₂Me₃Si, 3.2), 349 (4.8), 266 (44), 253 (26), 142 (43), 73 (100). Anal. (C₁₁H₁₇N₂O₃Cl) C, H, N.

N-(γ, γ -Dicarbethoxy- γ -phthalimidobutyl)-m-(benzyloxy)aniline (18b). Aldehyde 16 (12 g, 31.5 mmol), m-(benzyloxy)aniline (18.8 g, 94.5 mmol), and m-(benzyloxy)aniline hydrochloride (7.5 g, 31.5 mmol) were dissolved in 200 mL of absolute MeOH. To the solution was added 2.0 g (31.5 mmol) of NaBH₃CN in small portions. The clear yellow solution was stirred at room temperature for 2 days and acidified with concentrated HCl to pH 2. The mixture was evaporated to dryness under reduced pressure. The solid mass was suspended in 100 mL of H₂O and basified with 6 N NaOH solution. The mixture was extracted with ether 3 times, and the ether extracts were combined, dried over Na₂SO₄, and evaporated to dryness. The crude product was purified by chromatography on a silica gel column and eluted with CHCl₃-EtOAc (9:1, v/v) mixed solvent. Fractions with R_f 0.65 on silica gel plate (same solvent system) were pooled and evaporated to dryness to yield 11 g (64%) of yellow oil. Samples for elemental analyses were purified by preparative TLC: NMR (CDCl₃) δ 1.22 (6 H, t, CH₃, J = 3.5 Hz), 1.75 (2 H, CH₂, m), 2.60 (2 H, CH₂, m), 3.08 (2 H, t, NH₂, J = 3.0 Hz), 4.24 (4 H, q, OCH₂, J = 3.5 Hz), 4.92 (2 H, s, OCH₂), 6.15 (3 H, m, aromatic), 6.98 (1 H, m, aromatic), 7.25 (5 H, s, aromatic), 7.65 (4 H, m, aromatic); mass spectrum, m/z (relative intensity) 544 (M⁺, 56), 212 (100), 122 (12), 104 (8.0), 91 (75). Anal. (C₃₁H₃₂N₂O₇) C, H, N.

N-(γ,γ-Dicarbethoxy-γ-aminobuty])-*m*-(benzyloxy)aniline (19b). This compound, a yellow oil, was prepared on a 5.5-g scale (95%) from 18b by the same procedure used for 19a: mp for the dihydrochloride 170 °C (decomposed); NMR (CDCl₃) δ 1.22 (6 H, t, CH₃, *J* = 3.5 Hz), 1.90 (4 H, m, CH₂CH₂), 2.65 (3 H, s, NH₂, NH), 3.06 (2 H, t, CH₂, *J* = 3.0 Hz), 4.15 (4 H, q, OCH₂, *J* = 3.5 Hz), 4.95 (2 H, s, OCH₂), 6.18 (3 H, m, aromatic), 6.95 (1 H, m, aromatic), 7.30 (5 H, s, aromatic). Anal. (C₂₃H₃₀N₂O₅·2HCl-0.5H₂O) C, H, N, Cl.

 $N^{\overline{\delta}}$ -(*m*-Hydroxyphenyl)ornithine (1c). Amine 19b (9.5 g, 22.9 mmol) was refluxed in 100 mL of 0.5 N KOH for 4 h. After the solution cooled, the precipitates were collected and washed with a small amount of ethanol. The potassium salt was dissolved in 20 mL of water, acidified with concentrated HCl, and refluxed for 1.5 h. The solvent was evaporated to dryness, and the residue was suspended in 30 mL of MeOH and filtered to remove the potassium chloride. The filtrate was again evaporated to dryness, and the residue was dissolved in 30 mL of concentrated HCl and stirred at room temperature for 3 h. The reddish solution was extracted with CHCl₃ twice, and the aqueous layer was evaporated to dryness. The crude product was purified on a C_{18} reverse-phase column, using H_2O as eluent. The front band, $R_f 0.55$ (H_2O), fractions were pooled and lyophilized to give 0.7 g'(21%) of the final product, 1c. This compound is very hygroscopic and gives no specific melting point: mass spectrum (as $4Me_3Si$), m/z (relative intensity) 512 (M⁺, 22), 497 (M - CH₃, 1.7), 395 (M -CO₂Me₃Si, 3.1), 266 (33), 253 (17), 142 (66), 73 (100). Anal. $(C_{11}H_{18}N_2O_3Cl_2\cdot^2/_3H_2O)$ C, H, N, Cl.

In Vitro Growth Assays. A. Melanotic Melanoma (M_2R) and Amelanotic Melanoma (A_2) . Toxicity was assayed by growth inhibition of two melanoma cell lines, M_2R and A_2 . M_2R is a melanotic cell line developed from B16 murine melanoma and was obtained from J. P. Mather.¹⁹ A_2 is an amelanotic cell line cloned from M_2R and has a 27-fold lower tyrosinase specific activity than M_2R^{16} as assayed by the tyrosine hydroxylation method of Pomerantz.²⁰

For growth studies, 1×10^4 cells suspended in growth medium (a 1:1 mixture of Dulbecco's modified Eagle's MEM and Ham's F-12 with 1.2 g/L of NaHCO₃, 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, and 10% fetal bovine serum) were seeded into 60-mm tissue-culture dishes. Incubations were at 37 °C in a humidified atmosphere of 5% CO₂ in air. After 24 h, the medium was aspirated off from above the cell monolayer and replaced with 4 mL of fresh medium containing various concentrations of the test substances. Growth was followed for at least 3 additional days by detaching the cells with trypsin and determining the cell number on a Coulter cell counter. Growth comparisons were made at a time when the control cultures had increased 8-fold, according to the following formula:

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growth (\% of control) =
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	01 00110101,		
_	net increase in cell number of test culture	~ 1	00
r	et increase in cell number of control culture	~ 1	.00

B. Human Epidermoid Carcinoma of the Nasopharynx and P-388 Murine Leukemia. These assays were carried out by the screening program of the Developmental Therapeutic Program of NCI.

In Vivo Antitumor Testing. Antitumor activity was determined as percent T/C values, with T/C $\geq 125\%$ defined as statistically significant. Dose-response studies were carried out for each compound according to published National Cancer Institute protocols.¹⁸ Treatment begins 24 h after intraperitoneal (ip) tumor implant on days 1–9 with intraperitoneal doses of the compound under investigation. Normal saline (0.9% NaCl) was used as a vehicle.

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Antitumor Agents. 2.¹ Bisguanylhydrazones of Anthracene-9,10-dicarboxaldehydes

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9,10-Anthracenedicarboxaldehyde bis[(4,5-dihydro-1H-imidazol-2-yl)hydrazone] (bisantrene, VI-1) showed anticancer activity in mice vs. both leukemias and solid tumors. Increases in life span vs. the following neoplasms were: P-388 leukemia, 137%; B-16 melanoma, 122%; Lieberman plasma cell tumor, >85%; colon tumor 26, 150%; Ridgway osteogenic sarcoma, 85%. There were significant numbers of long-term survivors. Both DNA and RNA synthesis were strongly inhibited. The drug was resistant to biodegradation and was bound strongly to tissues; in monkeys the half-life for disappearance from serum was 6 days. Related hydrazones were synthesized, and structure-activity relationships are discussed. Two routes to ring-substituted anthracene-9,10-dicarboxaldehyde intermediates were developed.

The compounds which have been shown to bind to DNA by intercalation² have generally been condensed tricyclic aromatics with at least one basic function. Since several of these compounds are active as antitumor agents,^{2,3} we synthesized and tested widely differing polycyclic aromatics with various basic side chains. We recently reported the synthesis and antitumor properties of one of these

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compounds.^{1,4} It has been named mitoxantrone (1) and is now undergoing clinical trials.⁵



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