# Agents with Potential Specificity against Melanotic Melanoma

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*Received September 14, 1981*

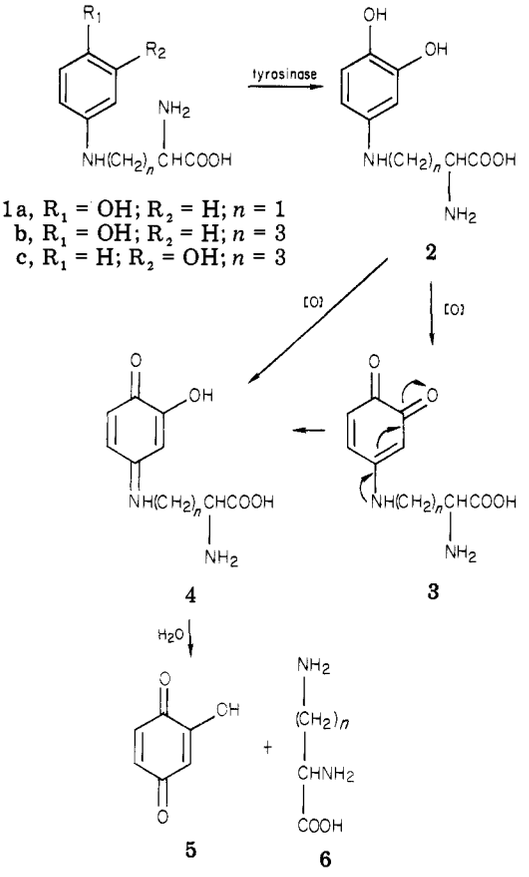
Agents were designed to exploit the high tyrosinase activity in melanotic melanoma relative to normal tissues. If specific tyrosinase activation of these agents occurred, the production of toxic metabolites in the melanoma cells would produce selective cell kill. Synthesis and antitumor activities of three new amino acids, **1a** [β-[(*p*-hydroxyphenyl)amino]alanine hydrochloride], **1b** [$N^{\delta}$-(*p*-hydroxyphenyl)ornithine hydrochloride], and **1c** [$N^{\delta}$-(*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 μM, the highest concentration tested. Similarly, compound **1b** exhibited better activity against P-388 ($ED_{50} = 9.5 \times 10^{-6}$ M) than **1a** ($ED_{50} = 3.2 \times 10^{-5}$ 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 $ED_{50}$ values in the range of 1.2 to $3 \times 10^{-4}$ 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**), $N^{\delta}$-(*p*-hydroxyphenyl)ornithine hydrochloride (**1b**), and $N^{\delta}$-(*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-1,4-benzoquinone (**5**) have not been studied, the antiviral and antibacterial activities of this compound are documented.[10,11] An analogous pathway for the generation of 2-hydroxy-4-iminoquinone has been reported[12] 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 Kreutz[13] to give ethyl formylhippurate (**8**). Treatment of **8** with *p*-(benzyloxy)aniline gave the enamine **11**, ethyl α-benzamido-β-

Scheme I

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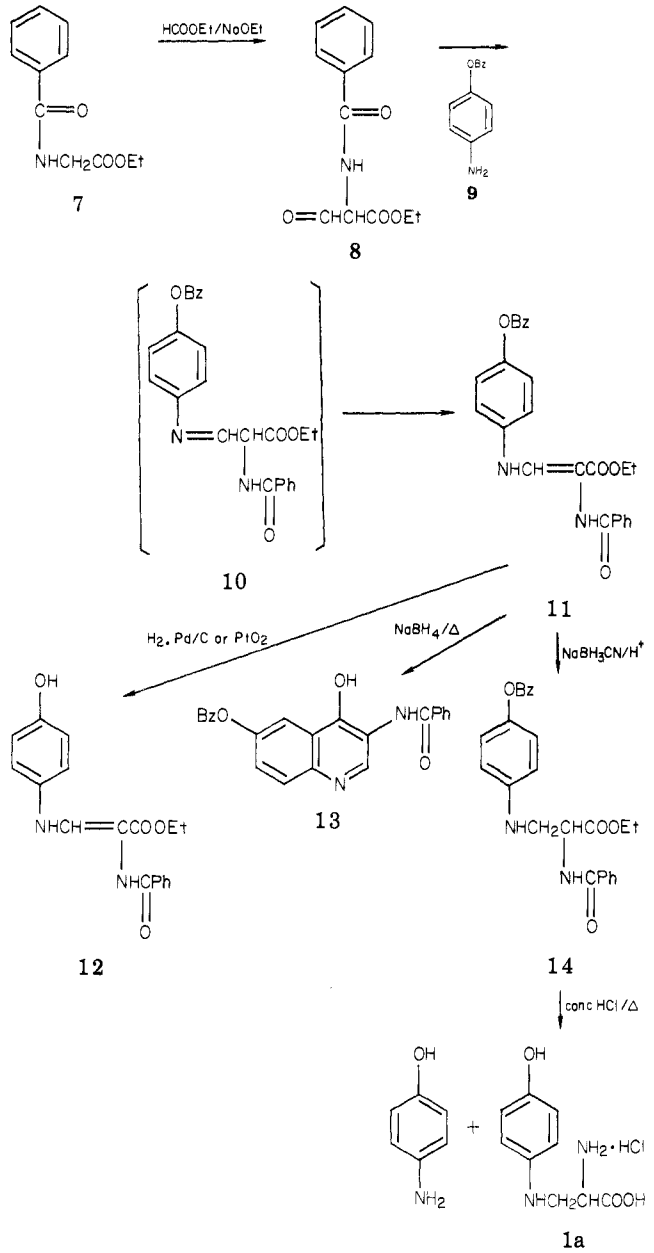
(1) K. P. Watts, R. G. Fairchild, D. N. Slatkin, D. Greenberg, S. Packer, H. L. Atkins, and S. J. Hannon, *Cancer Res.*, **41**, 467 (1981).
(2) V. Riley, Ed., "Pigment Cell", Vol. 1, S. Karger, Basel, München, Paris, London, New York, and Sydney, 1973.
(3) F. S. Vogel, L. A. K. Kemper, P. W. Jeffs, M. W. Cass, and D. G. Graham, *Cancer Res.*, **37**, 1133 (1977).
(4) S. M. Tiffany, D. G. Graham, F. S. Vogel, M. W. Cass, and P. W. Jeffs, *Cancer Res.*, **38**, 3230 (1978).
(5) F. S. Vogel, L. A. K. Kemper, K. Boekelheide, D. G. Graham, and P. W. Jeffs, *Cancer Res.*, **39**, 1490 (1979).
(6) P. C. Burger, L. A. K. Kemper, and F. S. Vogel, *Cancer Res.*, **39**, 1494 (1979).

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

Table I. In Vitro Antitumor Activities

| compd | cell lines | $ED_{50}$, $\mu$M |
|---|---|---|
| 1a | KB[a] | 140 |
| | PS[b] | 32 |
| | $M_2R$[c] | 24 |
| | $A_2$[d] | 54 |
| 1b | KB | 120 |
| | PS | 9.5 |
| | $M_2R$ | 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 |
| | $A_2$ | 120 |

[a] KB = human epidermoid carcinoma of the nasopharynx. [b] PS = P388 lymphocytic leukemia. [c] $M_2R$ = melanotic melanoma. [d] $A_2$ = amelanotic melanoma. [e] Reference 5.

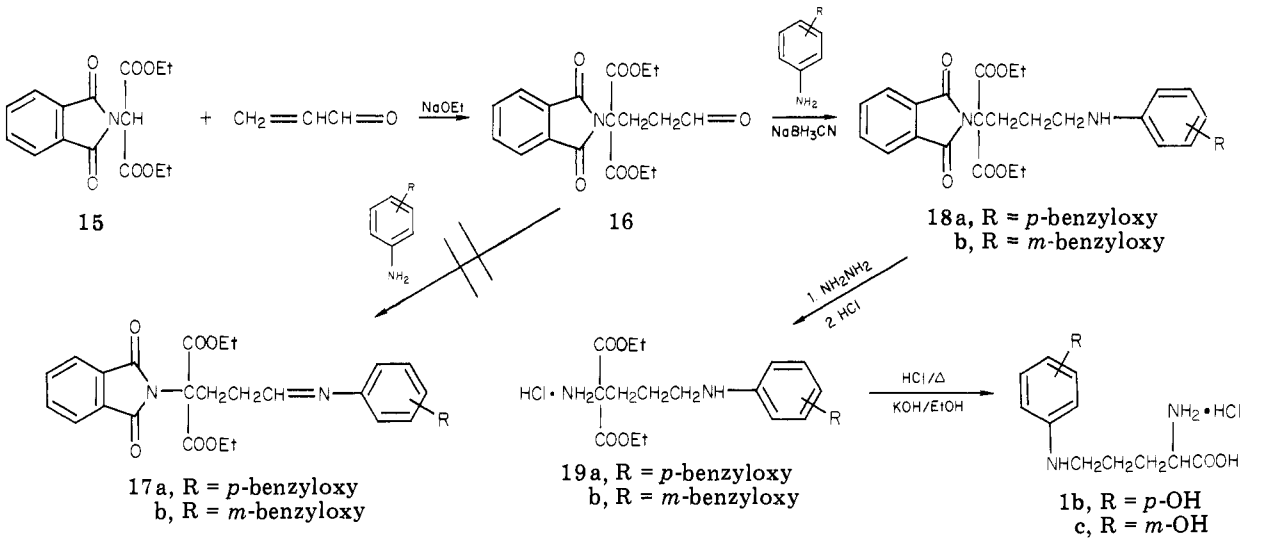
The conjugated double bond in **11** is resistant to both catalytic and chemical reductions. When compound **11** was subjected to catalytic reduction ($PtO_2$, Rh/C or Pd/C) using ethanol or acetic acid as solvent, the debenzylated product **12** was formed. Treatment of **11** with $NaBH_4$ in boiling 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 $NaBH_3CN$[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 $NaBH_4$ reduction; the second involved reductive amination of aldehyde **16** and *p*- or *m*-(benzyloxy)aniline using $NaBH_3CN$ 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

(7) K. Boekelheide, D. G. Graham, P. D. Mize, C. W. Anderson, and P. W. Jeffs, *J. Biol. Chem.*, **254**, 12185 (1979).
(8) P. D. Mize, P. W. Jeffs, and K. Boekelheide, *J. Org. Chem.*, **45**, 3540 (1980).
(9) A. Rosowsky, M. M. Wick, and S. H. Kim., *J. Med. Chem.*, **22**, 1034 (1979).
(10) M. Kuroya, N. Ishida, T. Shiratori, M. Miura, and Y. Yoshinari, *Yokohama Med. Bull.*, **4**, 73 (1953).
(11) G. D. Vecchio, V. D. Vecchio, and R. Argenziano, *Boll. Soc. Ital. Biol. Sper.*, **21**, 1 (1946).
(12) K. Boekelheide, D. G. Graham, P. D. Mize, and P. W. Jeffs, *J. Biol. Chem.*, **255**, 4766 (1980).
(13) E. Erlenmeyer, Jr., and A. Kreutz, *Justus Liebigs Ann. Chem.*, **337**, 236 (1904).
(14) L. C. Blaszczak and J. E. McMurray, *J. Org. Chem.*, **39**, 258 (1974).
(15) (a) R. F. Borch, M. D. Bernstein, and H. D. Durst, *J. Am. Chem. Soc.*, **93**, 2897 (1971). (b) O. A. Moe and D. T. Warner, *ibid.*, **70**, 2763 (1948).

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 **1a** against either cell line. No significant growth inhibitory activity was found for the *m*-hydroxy analogue **1c** at 100 $\mu$M, the highest concentration tested. In the same assay, the $ED_{50}$ of L-glutamic acid $\gamma$-(4-hydroxyanilide), the natural product isolated from *Agaricus bisporus*, was 97 $\mu$M against melanotic ($M_2R$) and 120 $\mu$M against amelanotic melanoma. These values are in agreement with reported results.[9] Thus, the new agents, **1a** and **1b**, are 4- to 8-fold more potent than the natural product. No significant selective activity of L-glutamic acid $\gamma$-(4-hydroxyanilide) against either melanotic or amelanotic melanoma was observed. Similarly, compound **1b** exhibited better activity against P-388 ($ED_{50}$ = 9.5 $\mu$M) than **1a** ($ED_{50}$ = 32 $\mu$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 $\times$ $10^{-4}$ 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 difference[16] 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 does not occur prior to the specific desired activation step. Further, the limited substrate specificity of mammalian tyrosinase which has been documented recently[17] should be taken into consideration in the design of new analogues of this class.

(16) T. R. Breitman and B. R. Keene, unpublished data.

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 $\gamma$-(4-hydroxyanilide)[a] | 400 | 124 |
| | 200 | 103 |
| | 100 | 120 |

[a] Reference 5.

Compounds **1a–c** were also tested against B-16 melanoma in vivo according to the published National Cancer Institute protocols[18] 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 $\gamma$-(4-hydroxyanilide) was reported[3] to possess marginal activity against B-16 melanoma, it showed no statistically significant (T/C $\geq$ 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 $\delta$ values relative to tetramethylsilane in $CDCl_3$ or 3-(trimethylsilyl)propionic acid sodium salt in $D_2O$. 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

(17) K. Boekelheide, D. G. Graham, P. D. Mize, and E. H. Koo, *J. Invest. Dermatol.*, **75**, 322 (1980).

(18) R. I. Geran, N. H. Greenberg, M. M. MacDonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep., Part 3*, **3**(2), 1 (1972).

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 silylated mixtures were separated on a 1.83 m × 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 silylation 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 silylation of **1a** and **1b** could be effected by heating the mixtures at 70 °C for 30 min, while smooth derivatization of **1c** without rearrangement required reaction at room temperature for several hours. Suitable aliquots (2–4 μL) of each silylation mixture were then analyzed by GC or GC/MS.

**Ethyl α-Benzamido-β-[*p*-(benzyloxy)anilino]acrylate (11).** Crude aldehyde **8**[13] (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 ($M^{+\cdot}$, 23), 370 (M – EtOH, 3.5), 325 (46), 279 (12), 105 (100), 91 (52), 77 (34); NMR ($CDCl_3$) δ 1.32 (3 H, t, $CH_3$, $J$ = 3.5 Hz), 4.26 (2 H, q, $CH_2$, $J$ = 3.5 Hz), 5.0 (2 H, s, $OCH_2$), 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 $H_2$ 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 ($M^{+\cdot}$, 57), 280 (M – EtOH, 3.6), 221 (48), 120 (32), 105 (100), 93 (6.7), 77 (39). Anal. ($C_{18}H_{18}N_2O_4$) C, H, N.

**Ethyl α-Benzamido-β-[*p*-(benzyloxy)anilino]propionate (14).** Compound **11** (20 g, 46 mmol) and $NaBH_3CN$ (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–$CHCl_3$, 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 $CHCl_3$ 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 ($M^{+\cdot}$, 8.5), 327 (26), 212 (54), 105 (100), 91 (47), 77 (34). Anal. ($C_{25}H_{26}N_2O_4$) 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 $C_{18}$ reverse-phase column chromatography (55–105 μm, Waters Associates, Inc.), using $H_2O$ as an eluent. A fraction collector was used to collect 10-mL aliquots, which were monitored by $C_{18}$ reverse-phase TLC. Fractions with $R_f$ 0.52 ($CH_3CN$–$H_2O$, 3:1, v/v) or 0.84 ($H_2O$) 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% $KMnO_4$ in 1 N NaOH was used to detect the spot on TLC; mass spectrum (as $4Me_3Si$), *m/z* (relative intensity) 469 (M – $CH_3$, 0.8), 367 (M – $CO_2Me_3Si$, 0.8), 291 (62), 266 (100), 219 (11), 193 (56), 178 (60), 147 (27), 101 (31), 73 (95). Anal. ($C_9H_{13}N_2O_3Cl$) 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 $CHCl_3$–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*-(γ,γ-Dicarbethoxy-γ-phthalimidobutyl)-*p*-(benzyloxy)aniline (18a).** Aldehyde **16** (12.0 g, 0.033 mol), *p*-(benzyloxy)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 $NaBH_3CN$ (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 ($M^{+\cdot}$, 16), 453 (77), 233 (20), 187 (36), 160 (33), 146 (54), 104 (43), 91 (100), 76 (33), 65 (30). Anal. ($C_{31}H_{32}N_2O_7$) C, H, N.

***N*-(γ,γ-Dicarbethoxy-γ-aminobutyl)-*p*-(benzyloxy)aniline (19a).** Compound **18a** (11.2 g, 20.6 mmol) and 85% $NH_2NH_2$ (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 $H_2O$ 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 ($CDCl_3$) δ 1.17 (6 H, t, $CH_3$, $J$ = 3.5 Hz), 1.70 (2 H, m, $CH_2$), 2.00 (2 H, m, $CH_2$), 2.48 (3 H, s, $NH_2$, NH), 3.00 (2 H, t, $CH_2N$, $J$ = 2.5 Hz), 4.10 (4 H, q, $OCH_2$), 4.85 (2 H, s, $OCH_2Ar$), 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. ($C_{23}H_{32}N_2O_5Cl_2$) C, H, N, Cl.

**$N^{\delta}$-(*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 $H_2O$, 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 $4Me_3Si$), *m/z* (relative intensity) 512 ($M^{+\cdot}$, 23), 497 (M – $CH_3$, 0.9), 395 (M – $CO_2Me_3Si$, 3.2), 349 (4.8), 266 (44), 253 (26), 142 (43), 73 (100). Anal. ($C_{11}H_{17}N_2O_3Cl$) 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 $NaBH_3CN$ 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 $H_2O$ and basified with 6 N NaOH solution. The mixture was extracted

with ether 3 times, and the ether extracts were combined, dried over $Na_2SO_4$, and evaporated to dryness. The crude product was purified by chromatography on a silica gel column and eluted with $CHCl_3$–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 ($CDCl_3$) $\delta$ 1.22 (6 H, t, $CH_3$, $J$ = 3.5 Hz), 1.75 (2 H, $CH_2$, m), 2.60 (2 H, $CH_2$, m), 3.08 (2 H, t, $NH_2$, $J$ = 3.0 Hz), 4.24 (4 H, q, $OCH_2$, $J$ = 3.5 Hz), 4.92 (2 H, s, $OCH_2$), 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. ($C_{31}H_{32}N_2O_7$) C, H, N.

***N*-(γ,γ-Dicarbethoxy-γ-aminobutyl)-*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 ($CDCl_3$) $\delta$ 1.22 (6 H, t, $CH_3$, $J$ = 3.5 Hz), 1.90 (4 H, m, $CH_2CH_2$), 2.65 (3 H, s, $NH_2$, NH), 3.06 (2 H, t, $CH_2$, $J$ = 3.0 Hz), 4.15 (4 H, q, $OCH_2$, $J$ = 3.5 Hz), 4.95 (2 H, s, $OCH_2$), 6.18 (3 H, m, aromatic), 6.95 (1 H, m, aromatic), 7.30 (5 H, s, aromatic). Anal. ($C_{23}H_{30}N_2O_5$·2HCl·0.5$H_2O$) C, H, N, Cl.

***N*$^\delta$-(*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 $CHCl_3$ 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 – $CH_3$, 1.7), 395 (M – $CO_2Me_3Si$, 3.1), 266 (33), 253 (17), 142 (66), 73 (100). Anal. ($C_{11}H_{18}N_2O_3Cl_2$·$^2/_3H_2O$) 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, $1 \times 10^4$ 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 $NaHCO_3$, 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% $CO_2$ 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:

$$\text{growth (\% of control)} = \frac{\text{net increase in cell number of test culture}}{\text{net increase in cell number of control culture}} \times 100$$

**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 ≥ 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.

(19) J. P. Mather and G. H. Sato, *Exp. Cell Res.*, **120**, 191 (1979).
(20) S. H. Pomerantz, *Science*, **164**, 838 (1969).

# Antitumor Agents. 2.[1] Bisguanylhydrazones of Anthracene-9,10-dicarboxaldehydes

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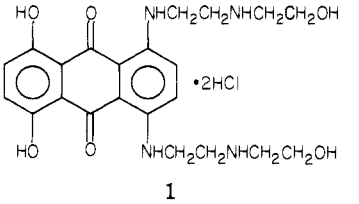
9,10-Anthracenedicarboxaldehyde bis[(4,5-dihydro-1*H*-imidazol-2-yl)hydrazone] (bisantrene, VI-1) showed anticancer activity in mice vs. both leukemias and solid tumors. Increases in life span vs. the following neoplasms were: P-388 leukemia, 137%; B-16 melanoma, 122%; Lieberman plasma cell tumor, >85%; 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 intercalation[2] 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 compounds.[1,4] It has been named mitoxantrone (1) and is now undergoing clinical trials.[5]

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