Synthesis and Antimetastatic Properties of Stereoisomeric Tricyclic Bis(dioxopiperazine) Analogues in a B16 Melanoma Model[1]

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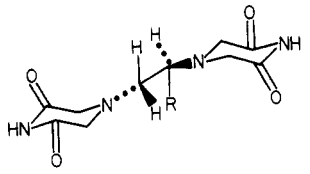
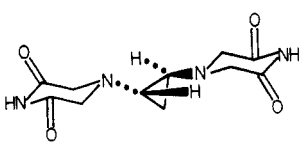
The synthesis for trans and cis tricyclic bis(dioxopiperazine)s **5** and **6** from pyrazine-2,3-dicarboxamide (**7**) is described. Stereoselective antimetastatic activity differences for these analogoues were observed following pretreatment of B16-F10 melanoma cells in vitro. Activities for these isomers were compared with selected intermediates, and the data are discussed in relation to previous results obtained with *cis*- and *trans*-cyclopropane analogues.

Antineoplastic drugs have cytotoxic effects on primary and metastatic tumors.[2] However, events leading to metastasis are not well understood. Drugs designed to have antimetastatic properties may play a significant role in cancer chemotherapy and lead to a better understanding of the mechanism of the metastatic process. Previous reports from these laboratories indicated that ICRF-159 (**1**) and its *cis*-cyclopropane analogue **4** reduced, whereas



1 (ICRF-159), R = Me
2 (ICRF-154), R = H



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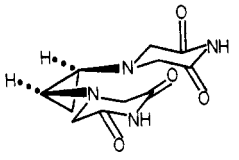

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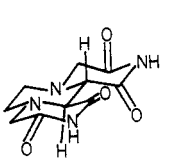
trans-**3** stimulated, metastasis in a hamster lung adenocarcinoma model.[3] Further studies revealed that treatment in vitro with low doses of *cis*-**4** and **1** inhibited lung colony formation of the murine B16 melanoma, whereas similar doses of the trans isomer stimulated lung colony formation.[4] Following pretreatment, colony formation in vitro, which was stimulated by both **3** and **4** correlated with the accelerated growth rates of the primary tumors in animals injected with these compounds. Bis(dioxopiperazine) **1** inhibited colony formation in vitro as well as in vivo.

Whereas compounds **3** and **4** exhibited stereoselective effects, little mechanistic work with these isomers is envisioned, since (1) more potent compounds are desired, (2) these analogues have markedly different solubilities which may influence their activities, (3) their multistep syntheses would virtually preclude ^{14}C labeling,[5] and (4) they are predictably unstable to catalytic ^{3}H-labeling procedures. In order to circumvent these problems, target tricyclic analogues *trans-anti-trans*-**5** and *cis-syn-trans*-**6** having a "cisoid" relationship of dioxopiperazine rings similar to those found in *cis*-**4** were constructed. Stereoisomers **5** and **6** are, in fact, related to ICRF-154 (**2**) and differ only by 1 mol of hydrogen in molecular weight. Furthermore, these compounds may be visualized as piperazine analogues of the ethylenediamine function in *cis*-**4** wherein the $-CH_2-$ of the cyclopropane ring is deleted and a -C–C- bond is formed between the C-2 and C-2′ positions of the diketopiperazine rings. In this article we describe synthetic methodology for these new tetraazaperhydrophenanthrenes (**5** and **6**) and our initial biological studies in the B16 melanoma model.



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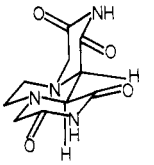

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Chemistry. Starting saturated diamide **9** was prepared from pyrazine-2,3-dicarboxamide (**7**) according to the method of Felder et al.[6] Under modified catalytic reduction conditions and workup, acid amide **8** was obtained, which upon reaction with ethyl bromoacetate yielded **10** and **11**, also tested as antimetastatic drugs. Alkylation of **8** using ethyl bromoacetate and K_2CO_3 in Me_2SO at 55 °C afforded triester **10** in 53% yield. When the alkylation was carried out at 100 °C for 20 h in the presence of benzyltriethylammonium chloride, diester imide **11** was obtained in 40% yield following chromatography on silica gel using

(1) A preliminary account of the chemistry of this series was presented to the Division of Medicinal Chemistry at the 181st National Meeting of the American Chemical Society in Atlanta, GA, Mar 29–Apr 3, 1981, by B. K. Trivedi, T. J. George, B. S. Zwilling, L. B. Campolito, N. A. Reiches, and D. T. Witiak. See "Abstracts of Papers", American Chemical Society, Washington, DC, 1981, Abstr MEDI 40.
(2) P. P. Carbone, *Cancer Res.*, 41, 1 (1981).
(3) D. T. Witiak, H. J. Lee, D. Goldman, and B. S. Zwilling, *J. Med. Chem.*, 21, 1194 (1978).
(4) B. S. Zwilling, L. B. Campolito, N. A. Reiches, T. J. George, and D. T. Witiak, *Br. J. Cancer*, in press.
(5) The Curtius reaction (using large quantities of azide) involving the preparation of the *cis*-1,2-cyclopropanediamine precursor never exploded in this laboratory, but others have experienced dangerous reactions [J. A. Landgrebe, *Chem. Eng. News*, 59(17), 47 (April 27, 1981)]. In our hands, the Curtius reaction leading to *cis*-1,2-cyclobutanediamine exploded violently, although no problems were encountered with either the *trans*-cyclopropane or cyclobutane isomers. Care should be exercized when carrying out Curtius reactions with 1,2-cycloalkylazides.
(6) Von E. Felder, S. Maffei, S. Pietra, and D. Pitre, *Helv. Chim. Acta*, 33, 888 (1960).

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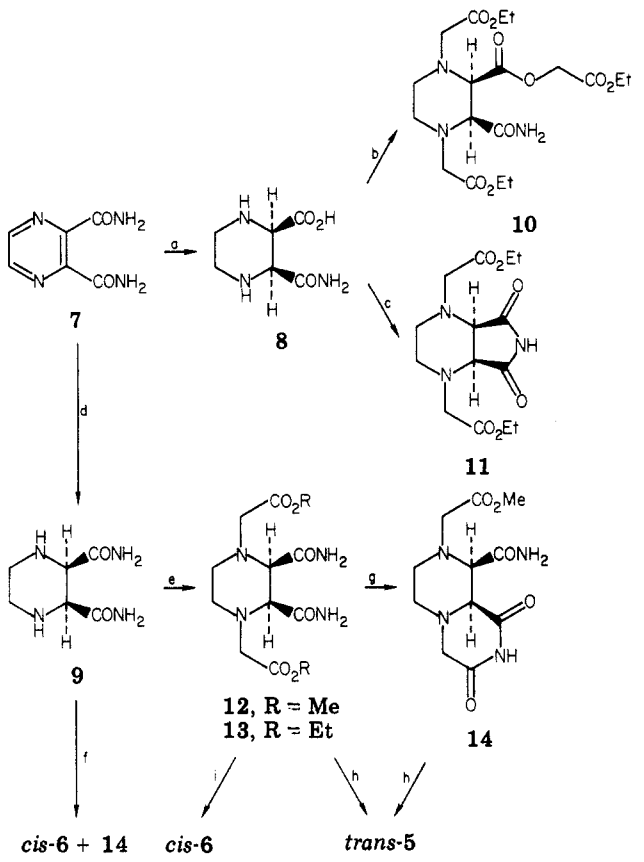
Scheme I[a]



[a] a = H_2O, 10% Pd/C, 50 psi; b = $BrCH_2CO_2Et$, Me_2SO, 55 °C, 3.5 h; c = $BrCH_2CO_2Et$, Me_2SO, 100 °C, 20 h; d = EtOH, 10% Pd/C, 50 psi; e = $BrCH_2CO_2Me$ for **12**, $BrCH_2CO_2Et$ for **13**, Me_2SO, room temperature; f = $BrCH_2CO_2Me$; Me_2SO; 65 °C; 6 h; g = silica gel-MeOH; h = NaOMe/MeOH; i = NaOEt/EtOH.

Et_2O/hexane as the eluant. Triester **10** likely was not produced in this reaction, in part owing to preferential formation of the thermodynamically more stable five-membered imide **11**. Furthermore, the quaternary ammonium salt moderately improved the yield of **11**.

Reaction of diamide **9** with either methyl or ethyl bromoacetate in Me_2SO at room temperature afforded diester diamide **12** and **13**, respectively, in 88 and 91% yield. Diester diamide **12** was quantitatively converted to **14** during chromatography on silica gel using MeOH as the eluant. Alkylation of **9** with methyl bromoacetate in Me_2SO at 65 °C for 6 h did not afford **12** but instead generated a mixture of bicyclic (**14**) and cis tricyclic (**6**) analogues in 51 and 14% yield, respectively.

Treatment of either **12**, **13**, or **14** with NaOMe/MeOH afforded only the trans isomer **5** in 67, 71, or 70% yield, respectively. No cis isomer was detected in the reaction mixture. However, when **12** or **13** was treated with NaOEt/EtOH under identical reaction conditions, the cis tricyclic isomer **6** was formed exclusively in 65 and 70% yield, respectively. Neither ester (**12** or **13**) could be converted to tricyclic compounds (**5** or **6**) in the absence of base. Exclusive formation of *trans*-**5** in NaOMe/MeOH was attributed to the complete solubility of reactants and products. Epimerization of *cis*-**6** to the thermodynamically more stable *trans*-**5** isomer in NaOMe/MeOH was not quantitative. Only a 60% yield of a cis/trans isomeric mixture was obtained in a ratio of approximately 1:3 (NMR analysis). Thus, epimerization of starting materials or intermediates mainly accounts for the exclusive formation of *trans*-**5** from **12** or **13** in NaOMe/MeOH. Possibly, biphasic base-catalyzed conversion of **12** or **13** to *cis*-**6** occurred without epimerization owing to insolubility of either reactants or products in NaOEt/EtOH.[7]

In addition to conformational analysis of reaction sequences, stereochemical assignments were based in part on ^1H NMR spectral analysis. Thus, in **10** the chemically nonequivalent methine proton resonance signals at δ 3.96 (CHCO) and 3.74 (*CH*$CONH_2$) with J = 3.5 Hz confirmed their cis relationship. In **9**, **11**, and **12** the methine proton resonance signals appeared as sharp singlets at δ 3.38, 3.91, and 3.54, respectively, owing to their chemical equivalency. Intermediate **8**, however, exhibited the expected cis coupling (J = 3.8 Hz) for the chemically nonequivalent methine protons. Interestingly, for **14** one methine proton resonance signal appeared at δ 3.95 (*CH*CONHCO), which is virtually identical with the resonance signal observed for the methine protons in **11** in D_2O. The methine proton α to the amide function in **14** has a resonance signal at δ 3.39 and in this regard is close to the analogous chemical shift for **9** and **12** in D_2O. However, in the case of **14**, the methine protons are chemically nonequivalent and cis coupling of J = 3.8 Hz was observed.

Inspection of Dreiding molecular models revealed the chiral trans-anti-trans isomer **5** to have a twofold axis of symmetry, whereas individual conformers of the cis-syn-trans isomer **6** are asymmetric.[8] However, the latter, on a rapid interconversion time scale, has an effective plane of symmetry rendering an achiral (meso) compound.[8] In fact, for **6**, as the temperature is increased from 25 to 80 °C the ^1H NMR spectrum in Me_2SO-d_6 simplifies. At room temperature a simpler ^1H NMR spectrum was observed for **5** when compared to **6**. In contrast to **6**, the ^1H NMR spectrum for **5** exhibited no change at the higher temperature in Me_2SO-d_6. In **5**, sharp methylene (δ 2.49, 4 H) and methine (δ 4.04, 2 H) proton resonance signals were observed for the central piperazine ring. Likewise, the methylene proton resonance signals of the dioxopiperazine rings showed a sharp AB quartet (δ_A 3.48, δ_B 3.41 with J_{AB} = 16 Hz). On the other hand, cis-**6** showed a broad singlet at δ 4.1 for the methine protons, and all methylene proton resonance signals were complex multiplets. Analogues **5** and **6** exhibited virtually identical fragmentation patterns in their mass spectra with m/e 252 (M^+).

Biological Results

The effects of a 24-h pretreatment of B16-F10 melanoma cells with 2, 20, and 100 μM concentrations of target tricyclic bis(dioxopiperazine)s *trans*-**5** and *cis*-**6** and selected analogues (**10**, **11**, and **14**) on experimental metastasis are shown in Table I. Following injection of cells into the tail vein of C57Bl/6J mice, both *trans*-**5** and tetraester **10** resulted in significantly decreased lung colony formation at all dose levels (experiment 1). Ester imide **11** exhibited no effect. In experiment 2, *trans*-**5** was compared with *cis*-**6** and bicyclic analogue **14**. Only *trans*-**5** significantly inhibited metastasis at all doses. Neither *cis*-**6** nor **14** had any effect. Colony inhibition in vivo was not a reflection of decreased colony formation in vitro. Out of approximately 100 cells, 50% formed colonies in vitro regardless of treatment.

(7) A reviewer has suggested that interconversion of **12** to **13** in NaOEt and **13** to **12** in NaOMe may be involved in selective formation of **6** and **5**, respectively, but further work is necessary to unravel the intricacies of these highly stereoselective processes.

(8) K. Mislow "Introduction to Stereochemistry", W. A. Benjamin, New York, 1965, pp 97–100.

Table I. Effect of Pretreatment of B16-F10 Melanoma Cells with Various Analogues on Experimental Metastasis

expt[a]	compd	dose:	mean no. of lung colonies 2 μM	20 μM	100 μM
1[b]	*trans*-5[c]		29.1 ± 24.8[d]	23.3 ± 19.7	65.7 ± 43.1
	10[c]		33.2 ± 37.2	43.4 ± 39.0	37.1 ± 28.2
	11[e]		159.7 ± 124	160.7 ± 126.8	142.5 ± 69.8
2[f]	*trans*-5[c]		39.1 ± 19.8	50.1 ± 23.7	50.2 ± 15.8
	14[e]		86.2 ± 31.8	101.3 ± 51.1	56.7 ± 36.8
	cis-6[e]		52.1 ± 13.3	96.1 ± 72.4	118.9 ± 88.3

[a] The control values for the saline-treated cells were not statistically different than those obtained for Me_2SO controls when Me_2SO is at the highest concentration employed. [b] Me_2SO control = 137.3 ± 64.2. [c] Significantly different from control as determined by Neuman-Keuls test. [d] Mean plus or minus SD. [e] Not significant. [f] Me_2SO control = 73.5 ± 34.8.

Discussion

Stereoselective antimetastatic activity differences for *trans*-**5** and *cis*-**6** are of particular interest. Since these isomers only differ in geometric orientation and have similar solubility properties, they may serve as probes for mechanistic studies. Furthermore, their relatively short syntheses should facilitate ^{14}C labeling. The antimetastatic properties observed for analogue **10** is unique, since all synthetic intermediates[4] to bis(dioxopiperazine)s **1** and **3–6** have no significant activity.

For bis(dioxopiperazine)s, antimetastatic activity seems to be dependent upon a preferred spacial orientation. Although the "cisoid" relationship of bis(dioxopiperazine) rings found in *cis*-**4** and *trans*-**5** appears to be important for antimetastatic activity, the inactivity of *cis*-**6**, also having "cisoid" bis(dioxopiperazine) rings, suggests that certain preferred conformations are required. Interestingly, Camerman and Camerman[9] have observed that the (+) enantiomer of **1** has the anti whereas the racemate has the eclipsed conformation in the crystalline state. It may well be, however, that the antimetastatic properties[4] of *dl*-**1** are a reflection of an eclipsed conformation similar to the juxtaposition of rings found in *cis*-**4** or *trans*-**5**.

Detailed dose-response studies with **1**, **4**, **5**, and **10** are in progress. Thus far, experiments involving pretreatment of B16-F10 melanoma cells in vitro indicate that **5** and **10** compare favorably with previous results[4] obtained using **1** at **2** and 20 μM concentrations of drug. Future testing in vivo using selected analogues observed to have antimetastatic effects by pretreatment in vitro of B16-F10 melanoma cells will be assessed for cytotoxic effects on primary and metastatic tumors using a tumor system which spontaneously metastasizes to the lung.

Experimental Section

Chemistry. All melting points are uncorrected and were taken on a Thomas-Hoover capillary melting point apparatus. Infrared spectra were recorded on a Beckman IR 4230 instrument. ^1H NMR spectra were determined on a Bruker 90 MHz instrument and mass spectra were obtained using a Dupont 491 mass spectrometer. Analyses were obtained from Galbraith Laboratories, Inc., Knoxville, TN.

Piperazine-2,3-dicarboxamide (9) was synthesized according to the method of Felder and co-workers[6] in 93% yield, mp 198–200 °C (lit.[6] mp 198–200 °C). Anal. ($C_6H_{12}N_4O_2$) C, H, N.

Dimethyl *cis*-2,3-Dicarbamoyl-1,4-piperazinediacetate (12). Methyl bromoacetate (2.93 g, 1.6 mL, 19.18 mmol) was slowly added (~10 min; using a syringe) to a suspension of **9** (1.5 g, 8.72 mmol) and anhydrous K_2CO_3 (1.32 g, 9.59 mmol) in 10 mL of Me_2SO under argon at room temperature. The reaction mixture was stirred at room temperature for 3 h and diluted with 150 mL of EtOAc. The inorganic salt was filtered, and the solvent was removed under reduced pressure. Me_2SO was removed at 0.3 mm (50 °C). The residue was dissolved in a minimum amount of absolute MeOH and diluted with 100 mL of EtOAc–hexane (1:1). The oil was solidified upon heating on a steam bath, filtered, and dried, affording 2.72 g of white solid. Recrystallization from MeOH–hexane yielded 2.42 g (88%) of a colorless compound (**12**): mp 172–173 °C; TLC (MeOH–EtOAc, 2:8) R_f 0.32; IR (KBr) 3400, 3300, 1730, 1650 cm^{-1}; NMR (D_2O) δ 3.63 (s, 6 H, OCH_3), 3.54 (s, 2 H, 2 >NC*H*CONH$_2$), 3.34 (s, 2 H, >NCH$_2$), 3.32 (s, 2 H, >NCH$_2$), 2.9–2.6 (m, 4 H, >NCH$_2$CH$_2$N<); MS (70 eV), *m/e* 299 ($M^+ - NH_3$), 240, 226. Anal. ($C_{12}H_{20}N_4O_6$) C, H, N.

***trans*-Tetrahydrodipyrazino[1,2-*a*:2′,1′-*c*]pyrazine-1,3,10,12(2*H*,4*H*,9*H*,11*H*)-tetrone (5). Method A.** To a suspension of diester diamide **12** (1.0 g, 3.16 mmol) in 6 mL of absolute MeOH was slowly added (~10 min) 1.8 mL of NaOMe (25% solution in absolute MeOH, 7.9 mmol) under argon at room temperature. The resulting colorless solution was heated at 60 °C for 4 h and stirred at room temperature for 16 h. The solvent was removed under reduced pressure, and the residual white solid was dissolved in 3 mL of H_2O. The aqueous solution was acidified (concentrated HCl, pH ~2), and the colorless solid was filtered, washed with 5 mL of cold H_2O followed by 5 mL of EtOH, and dried: yield 0.54 g (67%); mp 284–285 °C dec; IR (KBr) 3200, 3100, 1730–1690 (br) cm^{-1}; NMR (D_2O + NaOH) δ 4.04 (s, 2 H, 2 methine protons), 3.48 and 3.11 (AB q, 4 H, 2 >NCH$_2$CO, J_{AB} = 16.8 Hz), 2.49 (s, 4 H, >NCH$_2$CH$_2$N<); MS (70 eV), *m/e* 252 (M^+), 235, 181, 139. Anal. ($C_{10}H_{12}N_4O_4$) C, H, N.

Method B. A homogeneous solution of the diester diamide **13** (0.3 g, 0.872 mmol) in 3 mL of absolute MeOH containing 0.080 g of Na was heated at 85 °C for 6 h and at room temperature for 14 h. Following workup as previously described, 0.159 g (71%) of crystalline compound was obtained, which was identical in all respects with trans tricyclic compound **5** prepared from diester diamide **12**.

***cis*-Tetrahydrodipyrazino[1,2-*a*:2′,1′-*c*]pyrazine-1,3,10,12(2*H*,4*H*,9*H*,11*H*)-tetrone (6).** Methyl bromoacetate (2.22 g, 14.5 mmol) was slowly added to a suspension of diamide **9** (1.0 g, 5.8 mmol) and anhydrous K_2CO_3 (1.8 g, 13 mmol) in 6 mL of Me_2SO under argon at room temperature. The reaction was heated at 65 °C for 6 h, cooled to room temperature, and diluted with 70 mL of EtOAc. The inorganic salt was filtered, and the solvent was evaporated under reduced pressure. Me_2SO was removed at 0.3 mm (50 °C). The residue was dissolved in a minimum amount of absolute MeOH and diluted with 50 mL of EtOAc–hexane (1:1). The resulting solid was stirred in 30 mL of hot MeOH (steam bath), filtered, and dried: yield (0.21 g (14%); mp 282–284 °C dec; IR (KBr) 3200, 3100, 1730–1690(br) cm^{-1}; NMR (D_2O + NaOH) δ 4.1 (br s, 2 H, methine protons), 3.9–3.0 (m, 4 H, 2 >NCH$_2$CO), 3.0–2.4 (m, 4 H, >NCH$_2$CH$_2$N<); MS (70 eV), *m/e* 252 (M^+), 235, 181, 139. Anal. ($C_{10}H_{12}N_4O_4$) C, H, N.

Methyl *trans*-1-(Aminocarbonyl)octahydro-7,9-dioxo-2*H*-pyrazino[1,2-*a*]pyrazine-2-acetate (14). The filtrate resulting from the isolation of **6**, upon concentration under reduced pressure and recrystallization from MeOH–hexane, afforded 0.84 g (51%) of **14**: mp 214–216 °C dec; IR (KBr) 1730, 1690, 1650 cm^{-1}; NMR (D_2O) δ 3.95 (d, 1 H, >NCHCONH, J = 3.8 Hz), 3.66 (s, 3 H, CO_2CH_3), 3.55 (s, 2 H, >NC*H*$_2$CO$_2$CH$_3$), 3.54 and 3.2 (AB q, 2 H, >NC*H*$_2$CONH, J_{AB} = 18 Hz), 3.39 (d, 1 H, >NC*H*CONH$_2$, J = 3.8 Hz), 2.9–2.4 (m, 4 H, >NCH$_2$CH$_2$N<); MS (70 eV), *m/e* 284 (M^+), 252, 240, 225. Anal. ($C_{11}H_{16}N_4O_5$) C, H, N.

***cis*-3-(Aminocarbonyl)-2-piperazinecarboxylic Acid (8).** A suspension of **7** (5.0 g, 30 mmol) and 1.5 g of 10% Pd/C in 300

(9) A. Camerman and N. Camerman, personal communication.

mL of H_2O was hydrogenated at 50 psi for 20 h at room temperature. Filtration, followed by concentration of the solvent at 0.3 mm (50 °C), afforded 5.0 g of solid 8, mp 190–192 °C dec (lit.[6] mp 189–190 °C dec), which was not further purified but used as such in the conversion to 10: IR (KBr) 3600–3400, 1700 cm^{-1}; NMR (D_2O) δ 4.05 (d, 1 H >NC*H*CO_2H, J = 3.8 Hz), 3.73 (d, 1 H, >NC*H*$CONH_2$, J = 3.8 Hz), 3.4–2.9 (m, 4 H, >NCH_2CH_2N<), 2.93 (s, 2 H, $CONH_2$); MS (70 eV), m/e 173 (M^+), 156, 155.

Diethyl *cis*-2-(Aminocarbonyl)-3-[(2-ethoxy-2-oxoethoxy)carbonyl]-1,4-piperazinediacetate (10). Ethyl bromoacetate (6.4 g, 38 mmol) was slowly added to a suspension of 8 (3.0 g, 17 mmol) and anhydrous K_2CO_3 (2.65 g, 19 mmol) in 25 mL of Me_2SO under argon at room temperature. The reaction mixture was heated at 60 °C for 3.5 h and added to 50 mL of ice–H_2O. The solid organic material was filtered, dried, and crystallized from Me_2CO–Et_2O–hexane: yield 3.82 g (53%); mp 136.5–137 °C; TLC (EtOAc/Me_2CO, 9:1) R_f 0.65; IR (KBr) 3400–3200, 1730, 1650 cm^{-1}; NMR ($CDCl_3$) δ 7.9 (s, 1 H, CONH), 5.9 (s, 1 H, CONH), 4.7 and 4.43 (AB q, 2 H, $CO_2CH_2CO_2Et$, J_{AB} = 16 Hz), 4.16 (q, 2 H, $CO_2CH_2CO_2CH_2CH_3$, J = 7.3 Hz), 4.12 (q, 4 H, 2-CO_2CH_3, J = 7.2 Hz), 3.96 (d, 1 H, >$NCHCO_2^-$, J = 3.5 Hz), 3.74 (d, 1 H, >$NCHCONH_2$, J = 3.5 Hz), 3.62 and 3.42 (AB q, 2 H, >NCH_2CO_2Et, J_{AB} = 16.8 Hz), 3.5 and 3.3 (AB q, 2 H, >NCH_2CO_2Et, J_{AB} = 17 Hz), 3.0–2.6 (m, 4 H, >NCH_2CH_2N<), 1.21 (t, 9 H, 3 $CO_2CH_2CH_3$, J = 7.2 Hz); MS (70 eV), m/e 431 (M^+), 327, 254. Anal. ($C_{18}N_{29}N_3O_9$) C, H, N.

Diethyl *cis*-Hexahydro-5,7-dioxo-1*H*-pyrrolo[3,4-*b*]-pyrazine-1,4(4a*H*)-diacetate (11). Ethyl bromoacetate (4.24 g, 25 mmol) was slowly added (~10 min) to a suspension of 8 (2.0 g, 11 mmol), anhydrous K_2CO_3 (1.75 g, 12.7 mmol), and benzyl triethylammonium chloride (0.263 g, 1.15 mmol) in 20 mL of Me_2SO under argon at room temperature. The reaction mixture was heated at 100 °C for 20 h and the Me_2SO was removed at 0.3 mm (50 °C). The residue was dissolved in 15 mL of H_2O and the solution was extracted with EtOAc (4 × 75 mL). The organic extract was washed with brine (2 × 15 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The crude material (2.1 g) was chromatographed on 120 g of silica gel using three consecutive Et_2O–hexane mixtures of increasing polarity (5, 10, and 20% Et_2O): yield 1.5 g (40%); mp 114–115 °C; TLC (Et_2O–hexane, 8:2) R_f 0.32; IR ($CHCl_3$) 3400, 1730 cm^{-1}; NMR ($CDCl_3$) δ 8.26 (s, 1 H, NH), 4.16 (q, 4 H, 2 OCH_2CH_3, J = 7.2 Hz), 3.91 (s, 2 H, 2 >NCHCO), 3.74 (s, 4 H, 2 >NCH_2CO), 2.84 (s, 4 H, >NCH_2CH_2N<), 1.25 (t, 6 H, 2 OCH_2CH_3, J = 7.2 Hz), MS (70 eV), m/e 327 (M^+), 254, 212, 183. Anal. ($C_{14}H_{21}N_3O_6$) C, H, N.

Diethyl *cis*-2,3-Dicarbamoyl-1,4-piperazinediacetate (13). To a suspension of 9 (1.0 g, 5.81 mmol) and anhydrous K_2CO_3 (1.8 g, 13 mmol) in 5 mL of Me_2SO was added ethyl bromoacetate (2.42 g, 14.5 mmol) under argon at room temperature. The reaction mixture was stirred at room temperature for 20 h and diluted with 50 mL of EtOAc. The mixture was filtered and the filtrate was concentrated under reduced pressure. Me_2SO was removed at 0.3 mm (50 °C). The residue was dissolved in 20 mL of MeOH–EtOAc (1:1) and diluted with 100 mL of hexane. As the solution was standing at room temperature, 1.81 g (91%) of 13 crystallized: mp 120–121 °C; TLC (EtOAc–MeOH, 7:3) R_f 0.78; IR (KBr) 3400, 3200, 1720, 1650 cm^{-1}; NMR (Me_2CO-d_6) δ 7.5 (s, 2 H, $CONH_2$), 6.5 (s, 2 H, $CONH_2$), 4.12 (q, 4 H, 2 $CO_2CH_2CH_3$, J = 7.3 Hz), 3.6 (s, 2 H, 2 >NC*H*$CONH_2$), 3.39 (s, 4 H, 2 >NCH_2CO_2Et), 3.0–2.7 (m, 4 H, >NCH_2CH_2N<), 1.22 (t, 6 H, 2-$CO_2CH_2CH_3$, J = 7.3 Hz); MS (70 eV), m/e 344 (M^+), 327, 300, 283, 254, 252. Anal. ($C_{14}H_{24}N_4O_6$) C, H, N.

***cis*-Tetrahydrodipyrazino[1,2-*a*:2′,1′-*c*]pyrazine-1,3,10,12(2*H*,4*H*,9*H*,11*H*)-tetrone (6). Method A.** The diester diamide 13 (0.65 g, 1.88 mmol) was added to a solution of Na (0.173 g) in 7 mL of absolute EtOH at room temperature under argon. The resulting suspension in NaOEt was heated at 85 °C for 6 h, during which time the reaction mixture became turbid. Following stirring at room temperature for 14 h, the solvent was removed under redcued pressure, and the residual solid was dissolved in 3 mL of H_2O. The aqueous solution was acidified (concentrated HCl, pH 2), and the crystalline compound obtained upon cooling in the refrigerator was filtered, washed with 5 mL of cold H_2O followed by 5 mL of ethanol, and dried: yield 0.333 g (70%); mp 282–284 °C dec. This compound was identical in all respects with the cis tricyclic compound 6 prepared from diamide 9 as previously described.

Method B. A suspension of the diester diamide 12 (0.300 g, 0.949 mmol) in 3 mL of absolute EtOH containing 0.087 g of Na was stirred at 85 °C for 6 h and at room temperature for 14 h. Following workup as described above, 0.156 g (65%) of crystalline 6 was obtained.

Biological Methods. Male C57B1/6J mice were purchased from Jackson Laboratory, Bar Harbor, ME. Animals were housed 25 per cage, given food and water ad libitum, and used when 6–8 weeks of age. The B16-F10 melanoma was kindly provided by Dr. Isaiah J. Fidler (Frederick Cancer Center) and was maintained by in vitro culture.[10] Cultures were maintained for no more than 10 passages.

To assess effects of the various analogues on experimental metastasis, tumor cells were treated in vitro with 2, 20, or 100 μM concentrations of drug. Analogues were dissolved in Me_2SO at 1000 μM and diluted with tissue culture medium to the appropriate concentration. Controls consisted of tumor cells treated with Me_2SO or saline. Following a 24 h incubation, cells were removed from the monolayer by gentle scraping and washed in Hanks balanced salt solution (BSS). Cells were adjusted to 5 × 10^5/mL in Hanks BSS, and 0.2 mL was injected intravenously via the tail vein. Animals were sacrificed 14 days later by ether anesthesia, the lungs were removed, and the number of black nodules were enumerated with the aid of a dissecting microscope.

Statistical Analysis. Differences between the various doses of each compound were compared by one-way analyses of variance (ANOVA). The F test of the ANOVA was statistically significant for compounds 5 and 10. To determine the source of the significance, Newman–Keuls tests were performed.[11] This multiple comparison procedure tests the differences between all possible pairs of means in each experiment. By this procedure, it is possible to evaluate differences among doses, as well as differences from the control value.

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