Synthesis and Evaluation of Novel Spermidine Derivatives as Targeted Cancer Chemotherapeutic Agents

Peter A. Stark,*,†,‡ Brian D. Thrall,§,∥ Gary G. Meadows,§ and Mahmoud M. Abdel-Monem§

Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455, and Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, Pullman, Washington 99164-6510

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The utility of the spermidine moiety as the homing device for the selective delivery of chemotherapeutic and diagnostic agents into cancer cells was explored. Two spermidine analogs containing a cytotoxic agent were synthesized, *N*-[3,4-bis(benzyloxy)phenethyl]-N^{α}-(3-aminopropyl)-L-ornithinamide trihydrochloride, **1a** and *N*-[4-[bis(2-chloroethyl)amino]phenethyl]-N^{α}-(3-aminopropyl)-L-ornithinamide tetrahydrochloride, **1b**. These compounds were prepared from the fully protected spermidine molecule with a carboxyl group side chain, **8**. The ability of the polyamine cytotoxic agents to inhibit B16-BL6 melanoma cell growth in culture was examined. The effects of pretreatment with DFMO on the activity of the synthesized compounds was also studied. The IC_{50} values of compounds **1a** and **1b** were on the same order of magnitude as the control compounds, *N*-acetyldopamine and chlorambucil, respectively. The inhibitory activities of compounds **1a** and **1b** were not enhanced by pretreatment with DFMO, suggesting that depletion of intracellular polyamines did not enhance the activity of these compounds.

Introduction

All cells contain substantial amounts of at least one of the polyamines, putrescine, spermidine, and spermine.[1] Polyamines are a requirement for the optimum growth and replication of various cell types and are present in higher concentrations in rapidly proliferating cells.[1,2] Cellular polyamines are either synthesized intracellularly or transported from the extracellular environment. A key step in the biosynthetic pathway is the decarboxylation of ornithine to putrescine through the action of ornithine decarboxylase. Inhibition of ornithine decarboxylase depletes the level of intracellular polyamines, primarily putrescine and spermidine.[3-5] Many mammalian cells have a polyamine transport system which seems to be regulated by intracellular polyamine concentrations.[6,7] The transport system is also enhanced by stimuli that increase cell proliferation.[8]

This paper explores the use of the spermidine molecule as a carrier for the delivery of drugs or diagnostic agents to cancerous cells through the polyamine transport system in conjunction with an inhibitor of polyamine biosynthesis. This basic concept of selective delivery through the polyamine transport system has been examined previously, most notably through the work of Bergeron and Porter.[9-11] One study determined that the ability of spermidine derivatives to be taken up was dependent on the availability of the primary amines.[9] Consequently, the novel spermidine analogs proposed would attach a chemotherapeutic agent or diagnostic agent to the spermidine molecule through a linkage with the carbon backbone of spermidine (Figure 1). It was postulated that these polyamine analogs would achieve selective uptake in cancerous cells on the basis of the following observations: (1) the proposed spermidine analogs would be transported inside cells by the specialized polyamine transport system because of structural similarities between these analogs and spermidine; (2) the concentration of polyamines and their biosynthetic enzymes are enhanced in cancerous cells;[12] (3) the activity of the polyamine transport system is low in normal cells but is stimulated in rapidly growing cells;[7,13] (4) the depletion of intracellular polyamines, by using an inhibitor of ornithine decarboxylase for example, would increase the transport across the concentration gradient from the external medium into the cell; (5) the polyamine transport system exhibits preference for the

† University of Minnesota.
‡ Present address: Research Triangle Institute, P.O. Box 12194, Research Triangle Park, NC 27709.
§ Washington State University.
∥ Present address: Battelle Pacific Northwest Laboratory, Biology and Chemistry Department, Box 999, P7-53, Richland, WA 99352.

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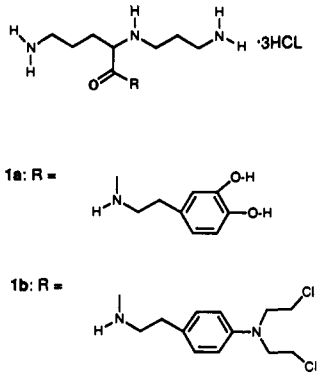


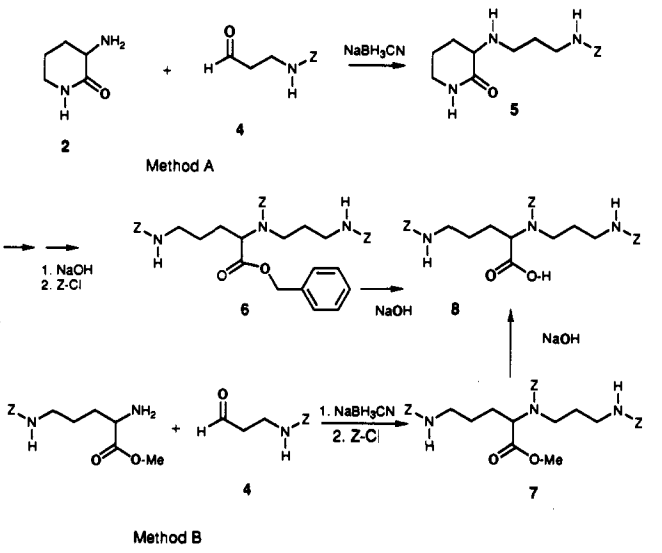
Figure 1.

naturally occurring polyamines in the order spermidine > spermine > putrescine.[9,14]

α-(Difluoromethyl)ornithine is a powerful inhibitor of ornithine decarboxylase and lowers polyamine concentrations in many cell types.[15-18] α-(Difluoromethyl)-ornithine is by far the most widely used inhibitor of ornithine decarboxylase used in both clinical and basic research. It has been shown that pretreatment with α-(difluoromethyl)ornithine enhances the uptake of naturally occurring polyamines, polyamine analogs, and compounds known to utilize the polyamine transport system.[10,14,19] Consequently, pretreatment with α-(difluoromethyl)ornithine has been used in conjunction with the spermidine analogs in this study.

In order to test our hypothesis of selective delivery it was useful to synthesize analogs whose active moiety produced their effects by different mechanisms of action. Catechols have been examined as antitumor agents in a variety of melanomas.[20-22] Nitrogen mustards have also received attention as cancer chemotherapeutic agents.[23] Since the mechanism of action for these two agents are different, they were selected as probes to investigate the ability of the spermidine molecule to deliver active agents to cancerous tissue. Linking the desired active agents to the spermidine molecule via an amide bond gave the desired compounds (**1a,b**), which were tested for their in vitro activity.

Scheme I



Results and Discussion

Chemistry. The synthesis of the key intermediate (**8**) was accomplished by two routes (Scheme I) both of which utilized ornithine and 3-aminopropanol. Method A converted ornithine into its methyl ester with anhydrous HCl in methanol, followed by cyclization to 3-aminopiperidone (**2**) using NaOMe according to a literature procedure.[24] The synthesis of 3-[(benzyloxycarbonyl)amino]-1-propanal (**4**) was accomplished from 3-[(benzyloxycarbonyl)-amino]-1-propanol (**3**) by oxidation employing a procedure of Swern.[25] Compound **3** was prepared from aminopropanol and benzyl chloroformate. The two portions were then combined by reacting the amine of compound **2** and the aldehyde of **4**, which formed an imine. Reduction of the imine using $NaBH_3CN$ gave intermediate **5**. Basic hydrolysis of the amide bond and protection of the amines, again with benzyl chloroformate, provided intermediate **6**. Basic hydrolysis of the benzyl ester of **5** gave the desired key intermediate **8**. Method B used δ-(benzyloxycarbonyl)-protected ornithine methyl ester and combined this with aldehyde **4** through reductive alkylation. Protection of the internal amine gave the intermediate **7**, and hydrolysis of the ester gave the desired intermediate **8**. Method B was advantageous in terms of yields and relative ease of preparation.

There is one chiral center in these spermidine analogs which corresponds to the α-carbon of ornithine. It was of interest to determine if the two synthetic routes lead to racemization and, if so, to what extent. This was accomplished by coupling intermediate **8** with optically pure (−)-phenylalanine methyl ester using a standard 1,3-dicyclohexylcarbodiimide (DCC) procedure and examining the products for the presence of diastereoisomers. HPLC was used to determine the ratio of the two diastereomers. Method A gave a 70:30 ratio of diastereoisomers and method B had a 95:5 ratio. The enhanced optical purity

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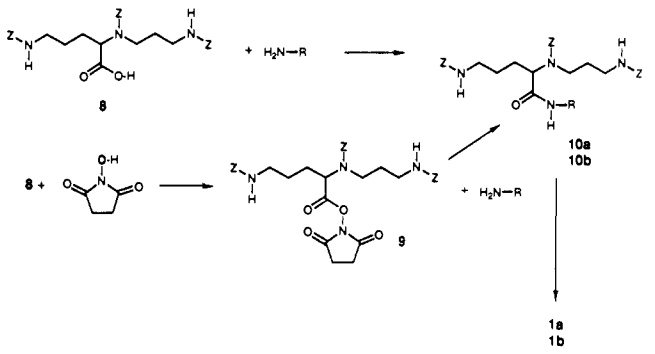
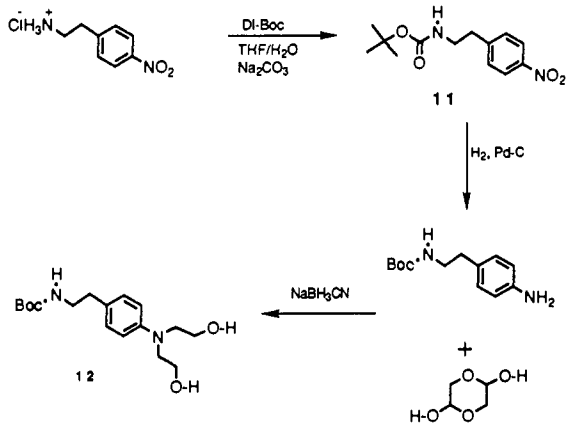
Scheme II



Scheme III



of method B was also apparent by comparing the optical rotation of intermediate **8** prepared from each of the routes.

Key intermediate **8** was linked to the active agents by two routes, which are depicted in Scheme II. The first route utilized familiar carbodiimide chemistry with either DCC or 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC). EDC proved more useful in that the urea byproduct was removed by aqueous extraction. The second route involved the synthesis of the *N*-hydroxysuccinimide ester (**9**), which was then reacted with the appropriate amine to form the desired amides. The second route was useful in that the active ester was a crystalline compound. Once the active agents were linked to the key intermediate, deprotection using hydrogenolysis afforded the desired compounds **1a** and **1b**.

The amine needed for the synthesis of **1a** was 3,4-bis-(benzyloxy)phenethylamine and was commercially available. The amine necessary for the synthesis of the nitrogen mustard (**1b**) was synthesized from 4-nitrophenethylamine as shown in Scheme III. The amino group was protected with *tert*-butyloxycarbonyl (BOC), giving **11**. The nitro group was then reduced using H_2 and Pd/C. Reductive alkylation using glycoaldehyde dimer gave the protected bis-hydroxyethyl intermediate **12**, which was deprotected and coupled to intermediate **9** prior to conversion of the hydroxyls to the corresponding chloro compound using thionyl chloride. Deprotection via hydrogenolysis in acidic MeOH gave **1b** as the HCl salt.

Biological Results

The effect of varying concentrations of α-(difluoromethyl)ornithine (DFMO) on the B16-BL6 melanoma cells was examined by studying growth inhibition in vitro and is shown in Table I. The results confirmed earlier work that DFMO inhibits cell growth.[6] The effect of DFMO on the spermidine content of the cells was also determined and is shown in Table I. Spermidine content was measured by HPLC analysis using the procedure of Verkoelen et al.[26] This procedure involved precolumn derivitization of the polyamines with benzoyl chloride followed by separation on a bonded column. These results show that the intracellular levels of spermidine decreased with DFMO treatment. The condition desirable for enhanced uptake of the spermidine derivatives is cell growth with depleted polyamines. In our study with B16-BL6 melanoma cells this condition was met at 0.25 mM DFMO.

Table I. Effect of DFMO on Cell Growth and Polyamine Content

DFMO concn (mM)	no. of cells/mL (× 10^5)	spermidine concn (nmol/10^6 cells)
0.0	24.3 ± 1.3[a]	2.07 ± 0.01[a]
0.1	21.8 ± 1.2	0.60 ± 0.01
0.25	19.4 ± 1.9	0.34 ± 0.03
0.5	10.9 ± 1.1	0.55 ± 0.05
0.75	13.3 ± 1.3	0.52 ± 0.04
1.0	14.8 ± 1.0	0.35 ± 0.02

[a] Mean ± SEM.

Table II. Effect of DFMO on [^{3}H]Spermidine Uptake into BL6 Cells

DFMO concn (mM)	cpm/million cells (±SE)	DFMO concn (mM)	cpm/million cells (±SE)
0.0	146 600 ± 20 723[a]	0.5	214 540 ± 23 491
0.1	221 610 ± 19 573	0.75	226 404 ± 34 822
0.25	219 910 ± 14 931	1.0	250 160 ± 12 249

[a] Mean ± SEM.

The ability of DFMO to increase the uptake of extracellular polyamines is of great importance if our spermidine derivatives are to have an analogous increased uptake. In order to test the ability of B16-BL6 melanoma cells to transport spermidine into the cells, the effect of varying concentrations of DFMO pretreatment on the uptake of [^{3}H]spermidine was examined. The results, shown in Table II, indicate that pretreatment of DFMO on B16-BL6 melanoma cells increases the uptake of [^{3}H]spermidine. In particular, at a concentration of 0.25 mM DFMO the uptake of [^{3}H]spermidine was significantly different from that of the control by Duncan's and LSD test, $p < 0.05$.

Compounds **1a** and **1b** were investigated for their ability to inhibit growth of B16-BL6 melanoma cells in vitro. *N*-Acetyldopamine, a catechol, and chlorambucil, a nitrogen mustard, were used for comparisons of **1a** and **1b**, respectively, due to their similarities to the active moieties. The IC_{50} value for the compounds were determined on B16-BL6 melanoma cells alone and after pretreatment with 0.25 mM DFMO. The results in Table III show that the effect of DFMO on the IC_{50} was minimal for all compounds. However, the activities of **1a** and **1b** were on the same order of magnitude as those of *N*-acetyldopamine and chlorambucil, respectively.

Conclusions

Compounds **1a** and **1b** produced inhibition of B16-BL6 melanoma cells growing in culture. The IC_{50} of these compounds were of the same order of magnitude as the

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Table III. IC_{50} Values, Summary of Data

compound	IC_{50} value alone	IC_{50} value with 0.25 mM DFMO
1a (catechol)	1.23 ± 0.01 mM[a]	1.31 ± 0.1 mM[a]
N-acetyldopamine	1.22 ± 0.04 mM	2.06 ± 0.06 mM
1b (mustard)	333.0 ± 9.6 μM	511.6 ± 28.3 μM
chlorambucil	220.3 ± 1.1 μM	279.8 ± 4.7 μM

[a] Mean ± standard deviation.

control compounds *N*-acetyldopamine and chlorambucil, respectively. The inhibitory activities of compounds **1a** and **1b** were not enhanced by pretreatment with α-(difluoromethyl)ornithine, suggesting that depletion of intracellular polyamines did not enhance the uptake of these compounds. Since pretreatment of B16-BL6 with α-(difluoromethyl)ornithine resulted in increased uptake of spermidine, the lack of increased activity of **1a** and **1b** after pretreatment with α-(difluoromethyl)ornithine suggests that these compounds were not transported by the spermidine transport system in these cells. It is not known, however, whether the inability of α-(difluoromethyl)-ornithine pretreatment to enhance the activity of compounds **1a** and **1b** is unique to the B16-BL6 cell line or if this behavior would be observed with other cells. It appears that the spermidine transport system in B16 cells has different characteristics than those in other cell lines. Indeed, paraquat, which has been shown to utilize the polyamine transport system in several cell lines, was not accumulated in B16 cells.[19] It is therefore of interest to examine the effects of α-(difluoromethyl)ornithine pretreatment on the cytotoxic activity of compounds **1a** and **1b** in cell lines other than B16-BL6.

Experimental Section

All chemicals and solvents used throughout the work were of reagent grade unless otherwise specified. The solvents used in the HPLC purifications and assays were of HPLC grade and filtered through a 0.45-μm Chromtech filter. All solvents were redistilled and, where needed, dry solvents were stored over 3-Å molecular sieves. Tetrahydrofuran and methylene chloride were routinely distilled from lithium aluminum hydride and phosphorous pentoxide, respectively. Thin-layer chromatographic (TLC) analyses were performed on silica gel GF or GHLF plates (250 μm, 10 × 20 cm uniscored, Analtech). TLC plates were visualized under short-wave UV light followed by spraying with 5% cerric sulfate spray reagent and heating over a hot plate at 120 °C. Flash chromatographic purification was accomplished using silica gel 60, 40–63 μm (230–400 mesh). Ion-exchange column chromatography purification was accomplished on Amberlite IRC-50, synthetic cation-exchange resin. Melting points were determined with a Thomas-Hoover melting point apparatus and are uncorrected. Optical rotation measurements were recorded using a Perkin Elmer 241 polarimeter. Proton magnetic resonance (NMR) spectra were made on a JEOL FX90Q instrument or a Nicolet NT 300 WB spectrophotometer with tetramethylsilane (TMS) as an internal standard except in aqueous solution where dioxane was the standard. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ and the results were within ±0.4% of the theoretical values. Mass spectra were obtained on a Finnigan 4000 mass spectrometer using NH_3 as the ionization gas. Fast atom bombardment (FAB) mass spectra were obtained on a VG 7070 EHF 11-250 data system. Accurate mass measurements were obtained from high-resolution mass spectrum analyses and were found to be greater than 98% pure by high-pressure liquid chromatography (HPLC). HPLC analyses were conducted on a cartridge column (5 μm, C8) from Phase Sep Inc., Norwalk CT, with a solvent system of MeOH/H_2O (65:35) with 10 mM hexanesulfonic acid, 2% potassium phosphate monobasic, adjusted to pH 4 with phosphoric acid. HPLC work was done at room temperature with a flow rate of 1.5 mL/min.

3-Aminopiperidone (2). L-Ornithine (2.5 g, 14.8 mmol) was stirred in MeOH (100 mL) and HCl gas was bubbled into the mixture. L-Ornithine was completely dissolved in 15 min and the reaction mixture was stirred for 1 h. The solvent was removed by rotary evaporation to give a white solid. The solid was redissolved in MeOH, and HCl gas was again bubbled into the solution. After repeating this procedure, NaOMe (1.6 g, 30.5 mmol) was added, and the mixture was stirred for 2 h and the solvent was removed. Compound **2** was obtained in 96% yield as a very hygroscopic tan solid and was used without further purification. IR: 3339, 2875, 1651, 1496, and 1314 cm^{-1} (lit.[24] IR 3300, 2800, 1650, 1490, and 1300 cm^{-1}). ^1H NMR ($CDCl_3$): δ 1.4–2.0 (m, 4 H, CCH_2C), and 2.9–3.1 (m, 2 H, CCH_2N, 1 H, α-CH). ^{13}C NMR ($CDCl_3$): 20.82, 29.32, 41.57, 50.83, and 174.7 ppm.

3-[(Benzyloxycarbonyl)amino]-1-propanol (3). 3-Amino-1-propanol (5.4 g, 81.8 mmol) in 50 mL of H_2O and 50 mL of saturated Na_2CO_3 was cooled to 0 °C. With stirring of this solution, benzyl chloroformate (12.3 g, 84.3 mmol) was added. The mixture was stirred for 1 h at 0 °C and then allowed to come to room temperature and stirred for an additional 2 h. The reaction mixture was extracted with EtOAc (3 × 100 mL). The organic extracts were collected and dried over $MgSO_4$. The solution was filtered and evaporated. The residue was crystallized from EtOAc/hexane to give **3** (18.1 g, 94% yield), as a white powder. Mp: 51–52 °C. ^1H NMR ($CDCl_3$): δ 1.61–1.80 (m, 2 H, $CH_2CH_2CH_2$), 3.41 (q, 2 H, $HNCH_2$), 3.73 (q, 2 H, CH_2OH), 5.13 (s, $CH_2C_6H_5$), and 7.36 (s, $CH_2C_6H_5$). IR: 2952, 2882, 1700, 1539, 1454, 1264, and 1039 cm^{-1}. Anal. ($C_{11}H_{15}NO_3$): C, H, N.

3-[(Benzyloxycarbonyl)amino]-1-propanol (4). A solution of CH_2Cl_2 (60 mL) and oxalyl chloride (2.4 mL, 25 mmol) was stirred in a 250-mL three-necked round-bottom flask equipped with two pressure-equalizing dropping funnels. This mixture was cooled to −65 °C, and the system was flushed with N_2. A mixture of DMSO (3.7 mL) and CH_2Cl_2 (8 mL) was slowly added over a period of 10 min. A solution of **2** (5.14 g, 25.0 mmol) in CH_2Cl_2 (15 mL) was added over a period of 5 min. The reaction mixture was stirred for 15 min. Et_3N (14 mL, 100 mmol) was added and the mixture allowed to come to room temperature. The solution was diluted with H_2O, the organic layer collected, and the aqueous layer reextracted with CH_2Cl_2 (2 × 25 mL). The combined organic extracts were dried over $MgSO_4$ and evaporated, and the product was purified by flash column chromatography on silica gel (acetone/hexane, 1:3) giving 4.21 g (84%) of **4**, which was crystallized from acetone/hexane to give rosettes. Mp: 45–46 °C. IR: 2952, 1707, 1532, 1454, and 1257 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.6 (s, 1 H, *HN*), 2.7 (t, 2 H, $CH_2C(O)H$), 3.4 (q, 2 H, $HNCH_2$), 5.08 (s, 2 H, $OCH_2C_6H_5$), 7.35 (s, 5 H, $CH_2C_6H_5$), and 9.8 (s, 1 H, *HC*(O)). ^{13}C NMR ($CDCl_3$, 77): 33.2, 42.6, 65.0, 128.1 (5C), 135, 154.8, 199.1 ppm. Anal. ($C_{11}H_{13}NO_3$): C, H, N.

3-[[3-[(Benzyloxycarbonyl)amino]propyl]amino]piperidine-2-one (5). A solution of **2** (3.38 g, 29.5 mmol) was stirred in $CHCl_3$ (100 mL) in a 250-mL three-necked round-bottom flask, fitted with a mechanical stirrer. Compound **4** (6.42 g, 30 mmol) and 4-Å molecular sieves (1 g) were added and the reaction mixture was stirred for 1 h. $NaBH_3CN$ (1.9 g, 30 mmol) was then added and the pH adjusted to approximately 6–7 with HCl/dioxane. The mixture was then stirred overnight. The solution was filtered and concentrated in vacuo. The residue was dissolved in EtOAc (100 mL) and extracted with 5% citric acid (50 mL) and 5% $NaHCO_3$ (50 mL). The organic layer was dried over $MgSO_4$ and filtered. The filtrate was concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel (EtOAc/MeOH, 98:2), which gave 3.16 g of **5** as an oil (35% yield). $[\alpha]^{25}_D$: +1.5° (*c* 0.48, MeOH). IR: 3339, 2952, 2875, 1651, 1496, and 1314 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.6-2.1 (m, 6 H, CCH_2C), 2.7 (t, 2 H, NCH_2C), 3.1–3.4 (m, 4 H, NCH_2C), 3.7 (t, 1 H, α-*CH*), 5.1 (s, 2 H, OCH_2Ph), and 7.36 (s, 5 H, CC_6H_5). High-resolution CI-MS: mass calculated for $C_{16}H_{24}N_3O_3$ 306.1847, found 306.1818.

Benzyl N^{α},N^{α}-Bis(benzyloxycarbonyl)-N^{α}-[3-[(benzyloxycarbonyl)amino]propyl]-L-ornithinate (6). To a solution of **5** (1.2 g, 1.52 mmol) in EtOH (25 mL) was added 1 N NaOH (5 mL) and the mixture was stirred at reflux. The reaction mixture was monitored by TLC ($CHCl_3$/MeOH/NH_4OH, 95:5:1) for the disappearance of **5**. It was complete in 8 h. The reaction

mixture was then neutralized with 1 N HCl, and the EtOH was removed by rotary evaporation. The resulting mixture was diluted with 25 mL of saturated Na_2CO_3 followed by the addition of benzyl chloroformate (2.01 g, 11.8 mmol). This mixture was stirred vigorously overnight and the resulting product partitioned from the aqueous phase. The reaction mixture was extracted with EtOAc (3 × 25 mL). The organic extracts were combined and dried over $MgSO_4$. The solution was filtered and concentrated in vacuo. The resulting crude product was purified by flash column chromatography (EtOAc/hexane, 2:3) to give pure **6** (0.62 g, 34% yield). $[\alpha]^{25}_D$: −10.6° (*c*, 0.49, MeOH). IR: 3339, 3030, 2945, 1700, 1525, 1461, and 1243 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.6–2.2 (m, 6 H, CCH_2C), 3.1–3.4 (m, 6 H, NCH_2C), 3.7 (t, 1 H, α-C*H*), 5.1 (s, 8 H, OCH_2Ph), and 7.36 (s, 20 H, CC_6H_5). Anal. ($C_{39}H_{43}N_3O_8$): C, H, N.

Methyl N^{α},N^{δ}-Bis(benzyloxycarbonyl)-N^{α}-[3-[(benzyloxycarbonyl)amino]propyl]-L-ornithinate (7). N^{δ}-Protected ornithine methyl ester (1.3 g, 4.2 mmol) was dissolved in 50 mL of $CHCl_3$ and stirred in a 250-mL three-necked round-bottom flask with an overhead mechanical stirrer with 4-Å molecular sieves (1 g). The flask was equipped with two pressure-equalizing dropping funnels containing **4** (0.88 g, 4.2 mmol) dissolved in $CHCl_3$ (10 mL) and $NaBH_3CN$ in 10 mL of chloroform. Compound **4** was added and the mixture was stirred for 5 h followed by the addition of the $NaBH_3CN$ solution. The reaction mixture was adjusted to approximately pH 6–7 by the addition of HCl/dioxane and stirred overnight. The solution was filtered, and the molecular sieves were washed with chloroform (3 × 50 mL). The crude product was concentrated in vacuo and dissolved in EtOAc (50 mL). The EtOAc solution was extracted with 5% aqueous sodium carbonate and then 5% aqueous citric acid. The organic solvent was removed, the crude product was redissolved in dioxane (50 mL), and saturated sodium carbonate (50 mL) was added. Benzyl chloroformate (0.76 g, 4.5 mmol) was then added, and the solution was stirred for 3 h. The dioxane was removed in vacuo, and the resulting oil was extracted from the aqueous layer with ethyl acetate (3 × 40 mL). The organic phase was dried over $MgSO_4$, and the solvent was evaporated to give a crude yellow oil. Flash chromatography on a silica gel and elution with EtOAc/hexane (40:60) afforded **7** (1.9 g, 75%) as a colorless oil. $[\alpha]^{25}_D$: −28.1° (*c* 0.42, MeOH). IR: 3339, 2945, 1700, 1525, 1447, 1250, 1018, and 737 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.5–2.1 (m, 6 H, CCH_2C), 2.9–3.4 (m, 6 H, NCH_2C), 3.6 (s, 3 H, OCH_3), 3.9 (t, 1 H, α-C*H*), 5.1 (s, 6 H, OCH_2Ph), and 7.3 (s, 15 H, CC_6H_5). Anal. ($C_{33}H_{39}N_3O_8$): C, H, N.

N^{α},N^{δ}-Bis(benzyloxycarbonyl)-N^{α}-[3-[(benzyloxycarbonyl)amino]propyl]-L-ornithinate (8). Compound **6** (0.45 g, 0.74 mmol) was dissolved in 25 mL of MeOH, and 2 mL of 1 N NaOH was added. The mixture was stirred for 3 h. The solution was diluted with 50 mL of citric acid, and the MeOH was removed by rotary evaporation. The product was extracted with EtOAc (3 × 25 mL). The organic extracts were combined and dried over $MgSO_4$. The crude product was concentrated in vacuo and purified by silica gel flash chromatography. The product was purified by flash column chromatography with an initial solvent of EtOAc/hexane followed by the addition of 1% AcOH to the solvent to give **8** (0.41 g, 0.69 mmol) for a yield of 93% from **6**. In an analogous manner **7** (1.35 g, 2.22 mmol) was converted to **8** (1.21 g, 92% yield). $[\alpha]^{25}_D$: −17.8° (*c* 0.54, MeOH). IR: 3339, 3036, 2948, 1698, 1532, 1461, 1257, 1138, 737, and 702 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.4–1.6 (m, 2 H, CCH_2C), 1.6–1.9 (m, 2 H, CCH_2C), 1.9–2.2 (m, 2 H, CCH_2C), 3.0–3.2 (m, 4 H, NCH_2C), 3.2–3.4 (m, 2 H, NCH_2C) 3.7 (t, 1 H, α-C*H*), 5.1 (s, 6 H, OCH_2Ph), and 7.3 (s, 15 H, CC_6H_5). High resolution FAB-MS: calculated for $C_{32}H_{38}N_3O_8$ 592.2658, found 592.2613. Anal. ($C_{32}H_{37}N_3O_8$): C, H, N.

Succinyl N^{α},N^{δ}-Bis(benzyloxycarbonyl)-N^{α}-[3-[(benzyloxycarbonyl)amino]propyl]-L-ornithinate (9). A solution of **8** (0.83 g, 1.4 mmol) and *N*-hydroxysuccinimide (0.17 g, 1.5 mmol) in dry THF (50 mL) was cooled in an ice bath. DCC (0.31 g, 1.5 mmol) was added to the stirred solution which was allowed to come to room temperature overnight. *N*,*N*′-Dicyclohexylurea was removed by filtration and the solvent was evaporated in vacuo. The crude product was dissolved in EtOAc (50 mL) and extracted with 5% citric acid (50 mL) and 5% $NaHCO_3$ (50 mL). The organic layer was dried over $MgSO_4$ and filtered, and the solvent was removed by rotary evaporation. The crude product was purified by silica gel flash column chromatography with a solvent system of EtOAc/hexane/AcOH (75:25:1) to give **9** as a clear oil. The pure active ester **9** (0.82 g, 85% yield) was crystallized from EtOAc/hexane. Mp: 52–53 °C. $[\alpha]^{25}_D$: −21.1° (*c* 0.88, MeOH). IR: 3339, 2945, 1743, 1707, 1532, 1257, and 1201 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.4–2.1 (m, 6 H, CCH_2C), 2.6 (s, 4 H, $COCH_2C$), 3.0–3.4 (m, 6 H, NCH_2C), 3.7 (t, 1 H, α-C*H*), 5.1 (s, 6 H, OCH_2Ph), and 7.3 (s, 15 H, $CH_2C_6H_5$). Anal. ($C_{36}H_{40}N_4O_{10}$): C, H, N.

***N*-[3,4-Bis(benzyloxy)phenethyl]-N^{α},N^{δ}-bis(benzyloxycarbonyl)-N^{α}-[3-[(benzyloxycarbonyl)amino]propyl]-L-ornithinamide (10a).** Compound **8** (94 mg, 0.13 mmol) was dissolved in dry THF (25 mL), and the stirred solution was cooled in an ice water bath. 3,4-Bis(benzyloxy)phenethylamine hydrochloride (58.8 mg, 0.16 mmol), Et_3N (50 μL), 1-hydroxybenzotriazole monohydrate (HOBt) (21.4 mg, 0.16 mmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (30.4 mg, 0.16 mmol) were added. The mixture was allowed to come to room temperature while stirring continued overnight. The solvent was removed by rotary evaporation and the residue dissolved in EtOAc (50 mL) and washed with water (50 mL). The organic layer was washed with 5% citric acid (50 mL) and 5% $NaHCO_3$ (50 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel (250–400 mesh) (EtOAc/hexane, 55:45) giving **10a** (72 mg, 75% yield) as a clear oil. $[\alpha]^{25}_D$: −21.1° (*c* 2.6, $CDCl_3$). IR: 3332, 3036, 2945, 1700, 1510, 1454, 1257, 1138, 737, and 695 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.3–2.0 (m, 6 H, CCH_2C), 2.6 (t, 2 H, CCH_2Ph), 3.0–3.5 (m, 8 H, NCH_2C), 4.1 (t, 1 H, α-CH), 5.1 (s, 10 H, OCH_2Ph), 6.6–6.9 (m, 3 H, $CC_6H_3(OR)_2$), and 7.3 (s, 25 H, CC_6H_5). Anal. ($C_{40}H_{45}N_4O_9$): C, H, N.

***N*-[3,4-Bis(benzyloxy)phenethyl]-N^{α}-(3-aminopropyl)-L-ornithinamide Trihydrochloride (1a).** Compound **9a** (45 mg, 0.06 mmol) was dissolved in methanol (2 mL) and placed in a hydrogenation bottle. To this solution were added 10 drops of dioxane, saturated with HCl, and 7 mg of 10% Pd/C. The flask was placed on a Parr hydrogenation apparatus and was filled with 40 psi of hydrogen and shaken overnight. The crude product was filtered through Celite, and the solvent removed by rotary evaporation. The product was recrystallized from MeOH/ether to give **1a** (22 mg, 81% yield) as a white solid that was very hygroscopic. $[\alpha]^{25}_D$: +0.67° (*c* 1.6, H_2O). IR: 3416, 2966, 1672, 1616, 1525, 1447, 1285, 1257, and 1110 cm^{-1}. UV: λ_{max} 204, ϵ_{max} 9000 (0.1 M HCl). ^1H NMR (D_2O): δ 1.43–1.69 (m, 2 H, CCH_2C), 1.82–1.97 (m, 2 H, CCH_2C), 2.0–2.16 (m, 2 H, CCH_2C), 2.82 (t, 2 H, CCH_2Ph), 3.0–3.4 (m, 6 H, NCH_2C), 3.7 (q, 2 H, NCH_2C), 3.82 (t, 1 H, α-C*H*), 6.92 (d, 2 H, *m*-C_6H_4OH), and 7.25 (d, 2 H, *o*-C_6H_4OH). FAB-MS: *m*/*z* 325 $(M + H - 3\ HCl)^+$, 181 $(M + H - CONHCH_2CH_2Ph)^+$, 433 $(M)^-$, and 217 $(M - H - Ph(O)_2)^-$. High-resolution FAB-MS: mass calculated for $C_{16}H_{29}O_3N_4$ 325.2223, found 325.2239.

***N*-[(*tert*-Butyloxy)carbonyl]-2-(4-nitrophenyl)ethylamine (11).** 4-Nitrophenethylamine hydrochloride (0.54 g, 2.6 mmol) and di-*tert*-butyl dicarbonate (0.58 g, 2.7 mmol) were dissolved in THF (50 mL). Saturated aqueous $NaHCO_3$ (50 mL) was added, and the mixture was cooled to 0 °C with an ice bath. The reaction mixture was stirred at 0 °C for 2 h and at room temperature overnight. The THF was removed and the product was extracted with EtOAc (2 × 30 mL). The organic layers were combined and dried over $MgSO_4$. The solution was filtered, and the product was recrystallized from EtOAc/hexane to give **11** (0.66 g, 96% yield) as fine needles. Mp: 94–95 °C. IR: 3332, 2980, 2931, 1700, 1518, 1349, 1257, 1166, and 857 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.4 (s, 9 H, CCH_3), 2.9 (t, 2 H, CCH_2Ph), 3.4 (q, 2 H, NCH_2C), 4.5 (bs, 1 H, N*H*), 7.4 (d, 2 H, *m*-$C_6H_4NO_2$), and 8.1 (d, 2 H, *o*-$C_6H_4NO_2$). Anal. ($C_{13}H_{18}N_2O$): C, H, N.

4-[Bis(2-hydroxyethyl)amino]-*N*-[(*tert*-butyloxy)carbonyl]phenethylamine (12). Compound **11** (0.16 g, 0.6 mmol) was dissolved in methanol (3 mL) and placed in a hydrogenation bottle with 10% Pd/C (20 mg). The bottle was filled with 40 psi of hydrogen and shaken for 2 h. The product was filtered and the solvent was removed in vacuo. The amine formed was used without further purification and dissolved in chloroform with 3-Å molecular sieves. Glycoaldehyde dimer (0.11 g, 0.9 mmol)

and $NaBH_3CN$ (80 mg, 1.2 mmol) were added, and the reaction mixture was stirred overnight. The solution was filtered, and the resulting solution was washed with 5% citric acid and 5% sodium bicarbonate. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The product was purified by flash column chromatography (EtOAc/hexane, 55/45), giving **12** (0.13 g, 71% yield) as a clear oil. IR: 3339, 2980, 2931, 1686, 1518, 1363, 1173, and 1046 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.4 (s, 9 H, CCH_3), 2.6 (t, 2 H, CCH_2Ph), 3.3 (q, 2 H, NCH_2C), 3.5 (t, 4 H, NCH_2C), 3.8 (t, 4 H, CCH_2OH), 6.6 (d, 2 H, *m*-C_6H_4), and 7.0 (d, 2 H, *o*-C_6H_4). FAB-MS: *m/z* 325 $(M + H)^+$, 269 $(M + C_4H_9)^+$. High-resolution FAB-MS: mass calculated for $C_{16}H_{28}N_2O_4$ 325.2127, found 325.2119.

***N*-[4-[Bis(2-hydroxyethyl)amino]phenethyl]-N^{α},N^{5}-bis(benzyloxycarbonyl)-N^{α}-[3-[(benzyloxycarbonyl)amino]propyl]-L-ornithinamide (10b).** Compound **12** (37 mg, 0.11 mmol) was stirred in a solution of dioxane/HCl. The solvent was removed, and the residue was stored in vacuo. The residue was dissolved in THF (35 mL) and Et_3N (1 mL) followed by the addition of the **9** (75 mg, 0.11 mmol). This mixture was stirred at room temperature for 3 h. The THF was removed by rotary evaporation, and the product was dissolved in EtOAc (25 mL). The organic solution was washed with 5% citric acid and 5% sodium bicarbonate. The organic layer was dried over $MgSO_4$ and filtered and the solvent was removed in vacuo. The residue was dissolved in dry CH_2Cl_2, thionyl chloride (1 mL) was added, and the mixture was stirred for 3 h. The solvent was removed, and the crude product was purified by silica gel flash column chromatography with an elution solvent of EtOAc/hexane (1:1), giving **10b** (69 mg, 73% yield). $[\alpha]^{25}_D$: −20.9° (*c* 2.6, MeOH). IR: 3332, 2938, 1700, 1525, 1454, 1250, 1138, 1025, 737, and 695 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.2–2.0 (m, 6 H, CCH_2C; 4 H, CCH_2Cl), 2.6 (t, 2 H, CCH_2Ph), 2.9–3.6 (m, 12 H, NCH_2C), 5.1 (s, 6 H, OCH_2-Ph), 6.5 (d, 2 H, *m*-C_6H_4), 6.9 (d, 2 H, *o*-C_6H_4), and 7.3 (s, 15 H, $CH_2C_6H_5$). FAB-MS: *m/z* 834 $(M + H - HCl)^+$, 798 $(M + H - 2HCl)^+$, 726 $(M + H - HCl - CH_2Ph)^+$, and 700 $(M + H - HCl - CO_2CH_2Ph)^+$. MS: calculated for $C_{44}H_{54}N_5O_7Cl_2$ 834.34003, found 834.3356.

***N*-[4-[Bis(2-hydroxyethyl)amino]phenethyl]-N^{α}-(3-aminopropyl)-L-ornithinamide Tetrahydrochloride (1b).** Compound 10b (34 mg, 0.04 mmol) was dissolved in methanol (2 mL), and 10 drops of dioxane/HCl were added. This mixture was placed in a 25-mL two-necked round-bottom flask equipped with a bubbler. Palladium on activated carbon (10% palladium) was added and hydrogen gas was bubbled through the system while stirring for 4 h. The product was filtered through Celite, and the solvent was evaporated in vacuo. The product was recrystallized from MeOH/ether to give **1b** (18 mg, 81% yield) as a extremely hygroscopic white powder. $[\alpha]^{25}_D$: +1.5° (*c* 0.2, 0.1 M HCl). UV: λ_{max} 209, ϵ_{max} 8512. IR (KBr): 3423, 2959, 1672, 1630, 1454, 1405, 1264, 1159, 1089, and 807 cm^{-1}. ^1H NMR (D_2O): δ 1.4–2.0 (m, 6 H, CCH_2C), 2.7 (t, 2 H, CCH_2Ph), 2.9–3.3 (m, 6 H, NCH_2C), 3.4–3.7 (m, 4 H, CCH_2Cl, 4 H, $PhNCH_2C$), 4.1 (t, 1 H, α-CH), 7.4 (bs, 2 H, Ph-*H*), and 7.5 (bs, 2 H, Ph-*H*). FAB-MS: *m/z* 466 $(M - H - 3HCl)^-$. High resolution FAB-MS: calculated for $C_{20}H_{35}N_5OCl_3$ 466.1907, found 466.1898.

Biological Methods. B16-BL6 Melanoma Cell Cytotoxicity Assay. A B16-BL6 melanoma variant isolated by Dr. Ian Hart was used in these in vitro studies.[27] The cells were cultured in Dulbecco's modified Eagles medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), sodium pyruvate, nonessential amino acids, L-glutamine, 2-fold vitamin solution, 5 μg/mL insulin, 100 units/mL penicillin, and 100 μg/mL streptomycin (complete medium). Cells were subcultured weekly at a split ratio of 1:4 and were maintained at 39 °C in a humidified atmosphere of 5% CO_2 in air. Cells were harvested using 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetracetic acid (EGTA) after washing the monolayers twice with Ca^{2+}- and Mg^{2+}-free Tyrodes' balanced salt solution (CMF).

The B16-BL6 melanoma cells were seeded at a density of 10^5 cells/well in a 6-well culture plate and allowed to attach and grow for 48 h. The cells were then treated with 0.25 mM DFMO in complete medium for 48 h. During the last 24 h of DFMO treatment, the cells were treated with appropriate drug. The drugs were removed, and the cells were washed twice with CMF to remove nonadherent cells. The remaining adherent cells were harvested with 10^{-4} EGTA and counted on an Elzone Model 280PC particle counter (Particle Data Inc.). Viability studies using the particle counter agreed with the studies using trypan blue exclusion as an indicator of viability (data not shown).

HPLC Analysis for Polyamine Content of the B16-BL6 Melanoma Cells. The cultured cells were washed with phosphate-buffered saline and centrifuged into a cell pellet. To the pellet were added 10 nmol per million cells of 1,6-hexanediamine (internal standard) and 1 mL of 0.3 M perchloric acid (PCA). To the clear supernatants were added 2 mL of 2 M sodium hydroxide and 10 μL of benzoyl chloride. The tubes were mixed vigorously and incubated for 30 min at room temperature. The incubation mixture was extracted with 2 mL of chloroform and washed with 2 mL of HPLC-grade water. The organic layer was evaporated under a stream of nitrogen and the residue resuspended in 100 μL of the mobile phase. The standard curve consisted of 10, 5, 1, 0.5, 0.1, and 0.05 nmol/mL of 0.3 M PCA and 10 nmol of internal standard. The polyamine standards were treated in the same manner as the culture supernatant.

Separations were performed at ambient temperatures using a Phase Sep C8 cartridge. Volumes of 10–100 μL of the residue dissolved in the mobile phase were injected onto the column. The mobile phase was methanol/water (65:35). Detection of the derivatives was accomplished by UV spectroscopy at a wavelength of 229 nm. The peaks were integrated with a Hewlett-Packard integrator. The samples were done in either duplicate or triplicate.

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