Thalidomide Metabolites and Analogues. 3. Synthesis and Antiangiogenic Activity of the Teratogenic and TNFα-Modulatory Thalidomide Analogue 2-(2,6-Dioxopiperidine-3-yl)phthalimidine[1]

Frederick A. Luzzio,*,† Alexander V. Mayorov,† Sylvia S. W. Ng,‡ Erwin A. Kruger,‡ and William D. Figg‡

Department of Chemistry, University of Louisville, 2320 South Brook Street, Louisville, Kentucky 40292, and Medicine Branch, National Cancer Institute, Bethesda, Maryland 20892

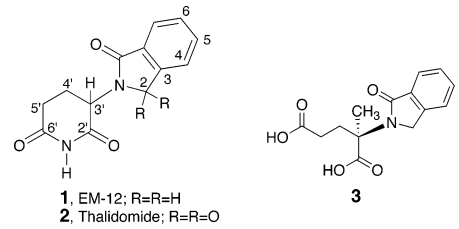
Received February 18, 2002

Versatile synthesis of the teratogenic, TNFα-modulatory, and antiangiogenic thalidomide analogue 2-(2,6-dioxopiperidine-3-yl)phthalimidine (**1**) and its direct antiangiogenic properties are described. With thalidomide or thalidomide derivatives as precursors, the synthesis involved either carbonyl reduction/thiation-desulfurization or carbonyl reduction/acyliminium ion reduction protocols. Compared to earlier studies with thalidomide, which was only active with microsomal treatment, **1** exhibited marginal inhibitory activity in the rat aortic ring assay, thereby demonstrating the requirement for metabolic activation.

Introduction

Angiogenesis is the development of new blood vessels from preestablished vasculature. The relevance of angiogenesis in tumor growth and metastasis has driven the study and development of antiangiogenic agents as cancer therapeutics.[2] Thalidomide and its analogues have attracted considerable attention as angiogenesis inhibitors, and questions remain about the similarity of the teratogenic and antiangiogenic mechanisms and how these compounds are activated in each of the biological responses. During early studies on the structure−activity relationships of thalidomide and recent studies of its antiangiogenic activity, the deoxythalidomide analogue 2-(2,6-dioxopiperidin-3-yl)phthalimidine (EM-12) or 2-deoxythalidomide (**1**, Chart 1) has emerged as the most biologically significant congener.[3] **1** exhibited a higher incidence of teratogenic effects than thalidomide (**2**, Chart 1) when tested in white New Zealand rabbits.[4a] Moreover, in the marmoset monkey, which is the thalidomide-sensitive primate model, **1** has been used as a probe to corroborate the activities of the individual enantiomers of thalidomide with its teratogenic effects.[5−7] Consequently, **1** and its analogues have seen increased use in bioassays where the mode of action and metabolic fate of thalidomide are still under investigation. The rationale for employment of **1** in biological studies is connected with its hydrolytic stability in the biological test system. The stability of the phthalimidine ring of **1** compared to the phthalimide ring of **2** contributes to a simpler hydrolytic profile so that biological disposition studies are unencumbered by a plethora of metabolites and their associated breakdown products.[8] The tumor necrosis factor-α (TNFα)-inhibitory activity of **1** has been demonstrated in lipopolysaccharide-stimulated (LPS) human peripheral blood mononuclear cell (PMBC) bioassays.[9] The configurationally fixed acyclic 3-methyl-*N*-phthalimidino analogue **3** (Chart 1) was found to inhibit B16BL6 pulmonary experimental metathesis in mice.[10] The common structural feature of compounds **1** and **3** (Chart 1) is the phthalimidine ring, which, in contrast to thalidomide, imparts increased hydrolytic resistance to the molecule and presumably facilitates more efficient transport to the molecular target.[11] The antiangiogenic activity of **1** was demonstrated in the basic fibroblast growth factor (bFGF)-induced rabbit cornea micropocket model and was found to be at least as potent as **2**.[12] The antiangiogenic activities of **1** and **2** were also investigated in the chicken chorioallantoic membrane (CAM) bioassay, and both compounds were inactive in the CAM model, thereby suggesting the requirement for metabolic activation of both compounds.[12] Finally, exposure of **2** to human or rabbit microsomes while incubating with rat aortic ring and human aortic endothelial cell cultures results in inhibition of angiogenesis. Control cultures conducted during the same study revealed that **2** incubated without microsomes was not inhibitory.[4b,13] The increased biological activity associated with analogues containing the phthalimidine ring together with reports that **2** exhibits a range of activities *without* exposure to an exogeneous metabolic activation system (MAS)[14] prompted us to explore new synthetic routes to **1** and analogues as well as to probe the activity of **1** when administered directly to an ex vivo angiogenesis assay system.

Chart 1



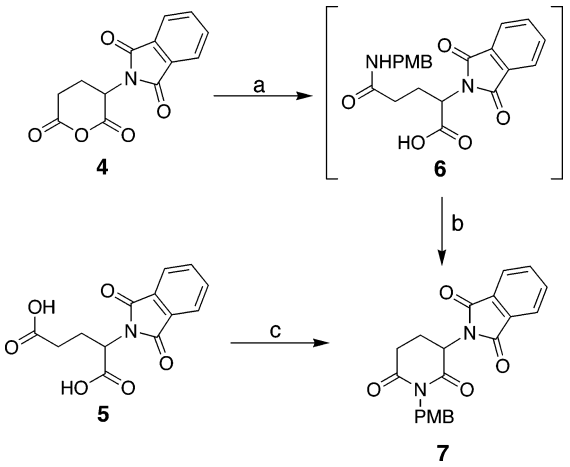
Results and Discussion

The synthesis commences with the preparation of *N*-parametoxybenzyl (PMB) thalidomide from (±)-3-

* To whom correspondence should be addressed. Phone: 502 852-7323. Fax: 502 852-8149. E-mail: faluzz01@athena.louisville.edu.
† University of Louisville.
‡ National Cancer Institute.

10.1021/jm020079d CCC: $25.00
Published on Web 07/30/2003

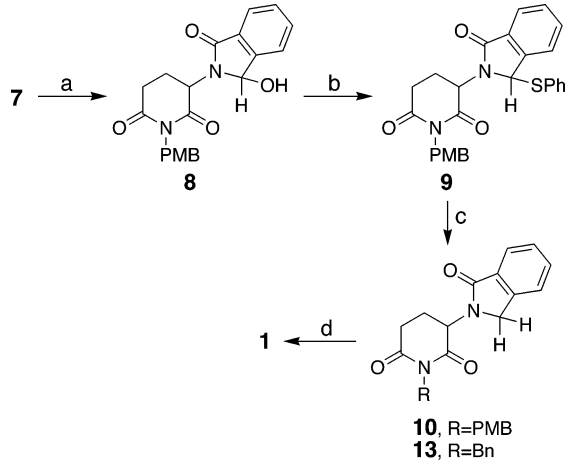
Scheme 1. Synthetic Scheme for (±)-*N*-PMB-thalidomide **7**[a]



[a] Reaction conditions: (a) 4-methoxybenzylamine, pyridine, 60 °C, 12 h; (b) $Ac_2O/(CF_3CO)_2O$, 70 °C, 12 h (51% from **4**); (c) 4-methoxybenzylamine, DCC, Et_3N, HOBt, CH_3CN, 50 °C, 15 h (54%).

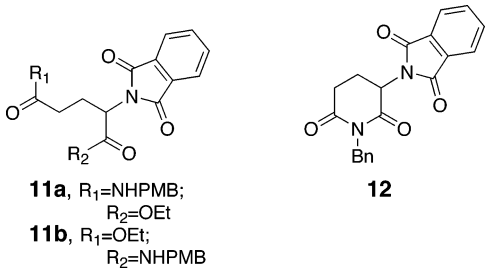
phthalimidoglutaric anhydride **4** or the corresponding glutaric acid **5** together with 4-methoxybenzylamine (Scheme 1). 4-Methoxybenzylamine facilitates the introduction of nitrogen as an "ammonia equivalent" in a formal sense when considering that the product is afforded at the end of the sequence by a deprotection step.[15,16] Two methods were used for *N*-(4-methoxybenzyl)glutarimide formation. Method A entailed the treatment of **4** with 4-methoxybenzylamine followed by direct treatment of intermediate **6** with acetic anhydride in the presence of a catalytic amount of trifluoroacetic anhydride to provide *N*-(4-methoxybenzyl)thalidomide **7** in 51% yield. Method B involved the exposure of (±)-2-phthalimidoglutaric acid **5** with triethylamine, 1-hydroxybenzotriazole (HOBt), 1, 3-dicyclohexylcarbodiimide (DCC), and 4-methoxybenzylamine at 50 °C for 15 h. Purification of the crude product under the same conditions as those of method A provided the *N*-PMB-3-phthalimidoglutarimide **7** in 54% yield. The conversion of the PMB-protected phthalimide **7** to the phthalimidine (isoindolinone) moiety of **10** was based on the chemoselective aluminum-amalgam-mediated reduction of the phthalimide group to the corresponding hydroxylactam, followed by phenylthiation and desulfurization, as briefly described in a preliminary communication (Scheme 2).[7] While the direct phthalimide to phthalimidine conversion may sometimes be facilitated with zinc/acetic acid,[18] the overall procedure described herein is milder and also avoids the employment of the less-selective hydridic reducing agents. Exposure of the *N*-PMB glutarimide **7** to freshly prepared aluminum amalgam (Al/Hg) in tetrahydrofuran/water followed by removal of the solid byproducts and reaction solvent system provided the crude hydroxylactam-substituted *N*-PMB-imide **8** without any concomitant reduction of the glutarimide ring. Direct treatment of hydroxylactam **8** with thiophenol and *p*-toluenesulfonic acid in dichloromethane resulted in quantitative phenylthiation, thereby furnishing the phenylthiolactam **9**. Reductive desulfurization of the sensitive phenylthiolactam **9** utilized Raney nickel in ethanol over 5 h at room temperature and provided the *N*-(4-methoxybenzyl)-

Scheme 2. Synthesis of **1** through *N*-PMB Derivatives[a]



[a] Reaction conditions: (a) Al(Hg), THF, H_2O, room temp, 3 h; (b) thiophenol, *p*-toluenesulfonic acid, CH_2Cl_2, room temp, 15 h; (c) Raney Ni, EtOH, room temp, 5 h, 80% (from **7**); (d) CAN, CH_3CN, H_2O, room temp, 2 h, 61%.

Chart 2

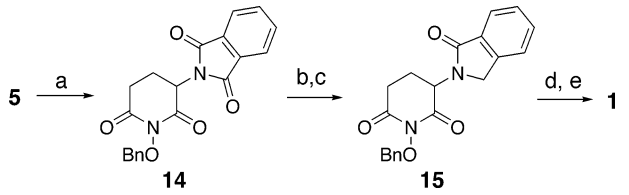


protected glutarimide (**10**) in 80% yield after purification by column chromatography. The age of the Raney nickel and the reaction time have a profound effect on the outcome of the desulfurization. Older lots of Raney nickel/ethanol combined with prolonged reaction times promoted ring-opening of the sensitive glutarimide ring, which led to the recovery of the *N*-PMB-glutamine and isoglutamine ethyl esters **11a** and **11b** (Chart 2).

Treatment of the *N*-PMB-protected phthalimidinoglutarimide (**10**) with ceric ammonium nitrate (CAN) in wet acetonitrile resulted in removal of the 4-methoxybenzyl group and furnished (±)-**1** as a white solid in 61% yield after purification by silica gel column chromatography. The employment of the benzyl group for protection of the glutarimide nitrogen was evaluated as a comparison to the PMB group. The *N*-benzyl derivative (**13**) was prepared in 75% yield by the aluminum amalgam/phenyl-thiation/desulfurization sequence starting with *N*-benzylthalidomide **12**, which in turn was prepared from benzylamine and **4**, albeit in low yield.[19] However, catalytic hydrogenation of **13** in tetrahydrofuran with 10% palladium on carbon under hydrogen (6 atm, 144 h) failed to effect any removal of the benzyl group as evidenced by 70% recovery of unreacted **13**.

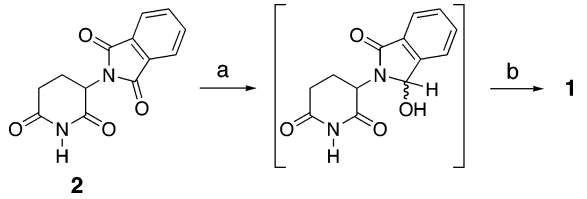
The second route to **1** employed a variation of a scheme reported by Galons for the asymmetric synthesis of thalidomide via *N*-BOC-glutarimide derivatives (Scheme 3).[20] An adaptation of the Galons scheme was of interest to us because its employment in conjunction with our reduction sequence could, in principle, be used in an asymmetric synthesis of **1** given that optically pure

Scheme 3. Preparation of **1** from *N*-Benzyloxythalidomide **14**[a]



[a] Reaction conditions: (a) $Ac_2O/(CF_3CO)_2O$, then pyr/$BnONH_3Cl$, then $Ac_2O/(CF_3CO)_2O$ (97%); (b) Al(Hg), THF, H_2O, room temp; (c) Et_3SiH, $(CF_3CO)_2O$, CH_2Cl_2, 0 °C, 2 h (41% from **14**); (d) Pd–C/MeOH /H_2, 24 h; (e) $BrCH_2COPh$, TEA, DMAP, CH_3CN, 4 h, room temp (21% from **15**).

Scheme 4. Direct Conversion of Thalidomide (**2**) to **1**[a]



[a] Reaction conditions: (a) Al(Hg), EtOH, H_2O, AcOH; (b) Et_3SiH, $(CF_3CO)_2O$, AcOH, 70 °C, 3 h (66%).

starting materials were used. Conversion of **5** to **14** (97%), which was effected through anhydride **4**, utilized benzyloxyamine hydrochloride. Reduction of NBO thalidomide **14** to the corresponding intermediate hydroxylactam was effected with Al/Hg similar to the conversion of **7** to **8**. The crude NBO hydroxylactam was not purified but directly treated with triethylsilane/trifluoroacetic acid[21] in dichloromethane to afford **15** as an amorphous white solid in 41% yield.

Removal of the *N*-benzyloxy group required a two-step process.[20] First, the benzyl group was cleaved with palladium on activated carbon under an atmosphere of hydrogen. Removal of the remaining *N*-hydroxyl group was effected by phenacyl ether formation and cleavage with phenacyl bromide/triethylamine/4-(dimethylamino)pyridine, thereby furnishing **1** in 21% yield after flash chromatography and crystallization. A more direct route to **1** was explored that did not involve various modes of N-substitution or protection and employed thalidomide itself as the starting material (Scheme 4). (±)-Thalidomide, prepared from 2-phthalimidoglutaric acid **5** by a modified procedure described by Helm,[3] was treated with in situ prepared aluminum amalgam in a mixture of ethanol/water/acetic acid (20:2:1) under reflux. As in Scheme 3, the crude corresponding hydroxylactam again was not purified but directly treated with a mixture of acetic/trifluoroacetic acid followed by heating with triethylsilane to afford **1** in 66% yield after recrystallization.

The rat aortic ring microvessel growth assay was used to evaluate the direct antiangiogenic properties of (±)-**1**.[13] The bioassay is a standard laboratory ex vivo preclinical screening experiment that is a modification of the procedure developed by Nicosia.[22] The technique was employed to measure the inhibition of new microvessel growth by **1** versus a positive control, carboxyamidotriazole (CAI). CAI is an effective angiogenesis inhibitor in the rat aortic ring model, thereby suppressing outgrowths through inhibition of calcium

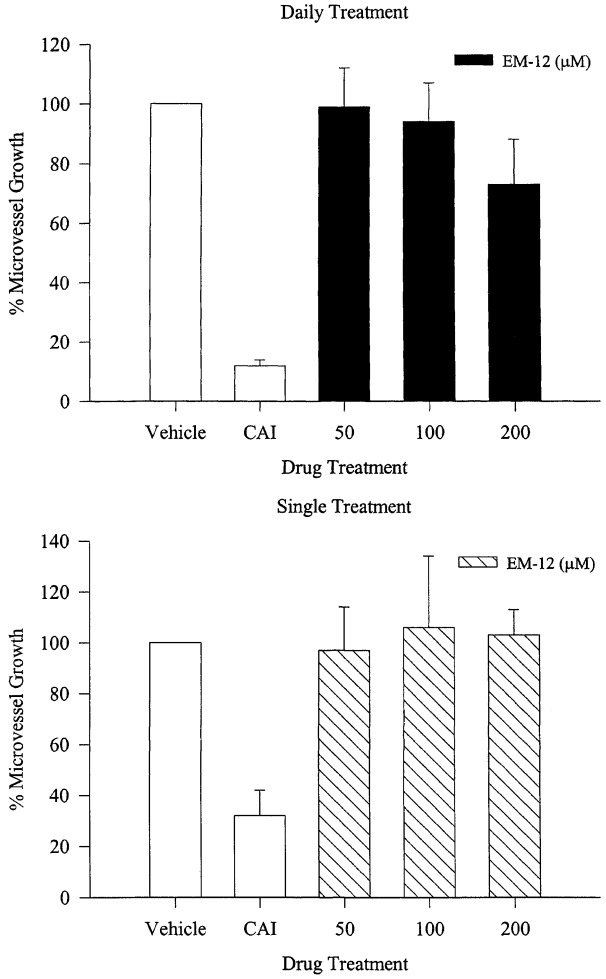


Figure 1. Top: inhibition of rat aortic ring microvessel growth (daily treatment for 4 days). Assays were done with medium (0.05% DMSO) and 30 μM carboxyamidotriazole (CAI)/medium as controls and 50, 100, and 200 μM **1**. Bottom: inhibition of rat aortic ring microvessel growth (single treatment over 4 days). Assays were done with medium and 30 μM carboxyamidotriazole (CAI)/medium as controls and 50, 100, and 200 μM **1** in medium (0.05% DMSO). The data represent the mean ± SEM (n = 4).

influx.[23] The inhibition experiments utilized working concentrations of **1** in endothelial basal media (EBM) and 0.05% DMSO vehicle, with EBM/0.05% DMSO as a control and CAI (30 μM) in EBM/0.05% DMSO.

The assays entailed both a single direct administration of the compounds to the rat aortic ring cultures on day 1 together with a separate regimen that entailed daily treatments of the cultures for 4 days. Digital photographic images of the cultures were recorded each day. The NIH Image program was used to quantify pixel density in the digital images by using a gray-scale analysis of background versus light pixels (outgrowths).[24] In turn, peripheral microvessel growth is quantitated and characterized. Moreover, nonspecific or inner-ring growth can be omitted and the method of quantitation allows for statistical analysis of the results. The title compound **1** was increasingly inhibitory at 100 and 200 μM (30%) when compared to the positive control, carboxyamidotriazole (CAI), which inhibited outgrowths by 90% as indicated by the bar graph (Figure 1, top). Microvessel outgrowth was not suppressed by a single treatment of **1** at 100 or 200 μM (Figure 1, bottom).

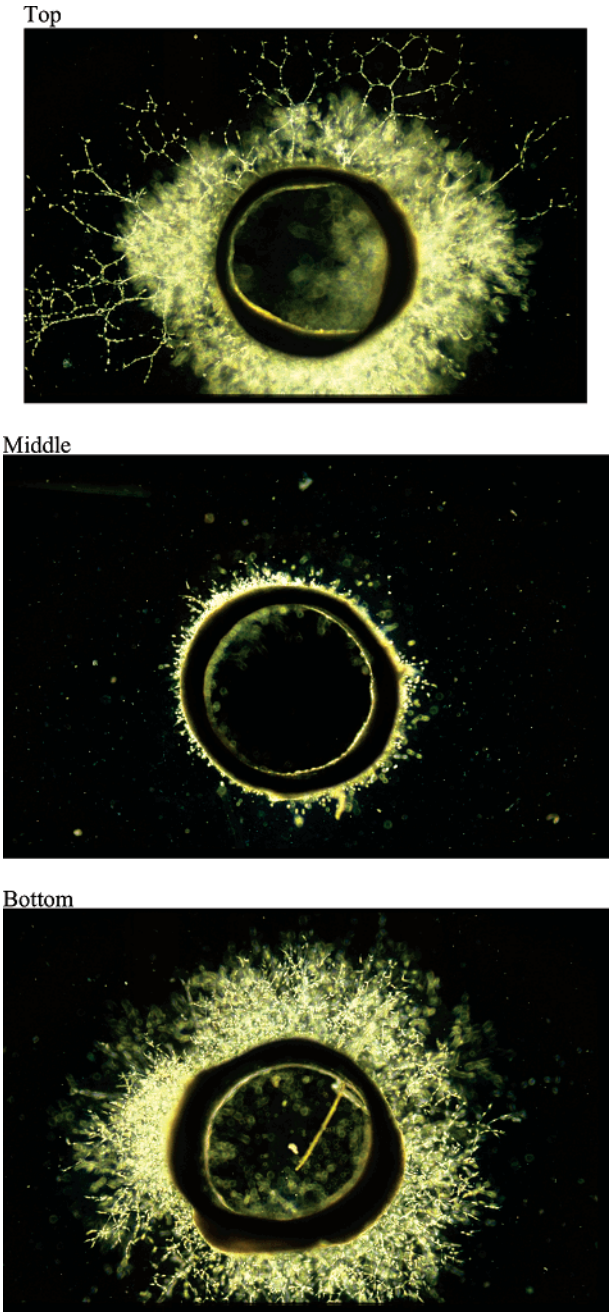


Figure 2. Images of rat aortic ring assays: (top) treatment of aortic ring culture with medium (0.05% DMSO); (middle) treatment of aortic ring culture with 30 μM carboxyamidotriazole (CAI)/medium; (bottom) treatment of aortic ring culture with 200 μM **1** administered daily for 4 days in medium (0.05% DMSO).

Photographic images of the aortic rings grown in growth media (Figure 2, top), in the presence of CAI (Figure 2, middle), and in the presence of 200 μM **1** administered daily (Figure 2, bottom) illustrate the relative inhibitory effects of the compounds. Compared to the direct daily administration of thalidomide in the rat aortic ring assay in which no inhibition of microvessel growth over the 40−200 μM concentration range was exhibited,[13] the slightly increased inhibitory activity by unactivated **1** is apparent. However, within the margin of error, the activities of **1** and **2** are comparable and the necessity for metabolic activation is demonstrated.

The activity of thalidomide or its analogues when bioassayed without in vivo or ex vivo metabolic activation is not without precedent. Direct administration of **2** significantly depletes glutathione and cysteine concentrations in New Zealand white rabbit whole embryo (WEC) cultures. In turn, depleted glutathione and cysteine levels interfere with normal redox cycling in the developing embryo, thereby leading to developmental malformations.[14]

Conclusion

We have detailed three serviceable synthetic routes to the thalidomide analogue (**1**). The direct conversion of thalidomide to **1** through the Al(Hg)/triethylsilane reduction provided the best yields. The mild, selective nature of all three routes makes them applicable to the preparation of the isoindolinone core structure and analogues of **1**, in racemic and optically active form as well as in suitable quantities for broad-range biological evaluation. The direct antiangiogenic activity of **1** in the rat aortic ring assay was found to be quite limited, and although of minimal statistical significance along the applied concentration range, an inhibitory trend was apparent. Within the framework of previous in vivo and ex vivo studies with thalidomide and in vivo studies with **1**, the lower range of inhibitory activity of **1** in the present ex vivo study further demonstrates the requirement for prior metabolic activation.

Experimental Section

General Procedures. Melting points are uncorrected. All the starting materials and products described are racemic mixtures. Dichloromethane and acetonitrile were distilled from CaH_2, and pyridine was distilled from barium oxide. Benzylic amines were fractionally distilled prior to use as reactants. All other reagents and solvents were ACS reagent grade and used as commercially available. Flash column chromatography was carried out using silica gel 60 (E. Merck 9385, 230−400 mesh).[24] Silica gel filtrations employed silica gel 60 (E. Merck 7734, 70−230 mesh). Celite filtrations employed Celite 521. Analytical thin-layer chromatographic (TLC) separations utilized 0.25 mm glass-backed silica gel plates (E. Merck 5715, silica gel 60 F_{254}). The TLC chromatograms were developed by dipping the plates in 2% anisaldehyde in ethanol or 2.5% phosphomolybdic acid in ethanol followed by heating (hot plate). Solutions, reaction mixtures, and chromatographic fractions were concentrated under vacuum using a standard rotary evaporator. Carbon signals marked with an asterisk represent methyl and methyne carbons as determined by APT experiments. High-resolution mass spectoscopic analyses (HRMS) were performed by the Nebraska Center for Mass Spectrometry, University of Nebraska, Lincoln and the University of California−Riverside Mass Spectrometry Facilty.

2-[1-(4-Methoxybenzyl)-2,6-dioxopiperidin-3-yl]isoindole-1,3-dione (7). Method A. (±)-3-Phthalimidoglutaric anhydride **4** (1.49 g, 5.76 mmol) was placed in an oven-dried round-bottom flask. Freshly distilled pyridine (8 mL) was added and then followed by 4-methoxybenzylamine (750 μL, 5.74 mmol). The reaction mixture was stirred at room temperature under an atmosphere of nitrogen (0.5 h) followed by heating (60 °C, 12 h). Pyridine was then removed under vacuum, which provided a syrupy residue. To the residue was added acetic anhydride (15 mL) followed by a catalytic amount of trifluoroacetic anhydride (100 μL), and the mixture was heated at 70 °C (12 h). Upon completion of the reaction, as determined by TLC, the volatile components were removed under vacuum followed by addition of ethyl acetate and filtration through a silica gel plug. The yellow filtrate was concentrated under vacuum and flash-chromatographed (hexanes/ethyl acetate, 1:1) to provide 1.11 g (51%) of *N*-PMB-

thalidomide **7** as an amorphous white solid. R_f = = 0.41 (hexane/ethyl acetate, 1:1). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.87 (m, J = 3.2, 5.5 Hz, 2H), 7.74 (m, J = 5.2, 5.5 Hz, 2H), 7.31 (d, J = 8.6 Hz, 2H), 6.8 (d, J = 8.6 Hz, 2H), 5.00 (d, J = 14 Hz, 1H), 4.96 (m, 1H), 4.82 (d, J = 14 Hz, 1H), 3.76 (s, 3H), 2.96 (m, 1H), 2.76 (m, 2H), 2.08 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 170.7, 168.6, 167.4, 159.0, 134.4*, 131.8, 130.4*, 128.9, 123.8*, 113.8*, 55.2*, 50.2*, 43.3, 32.1, 22.0. HRMS *m/z* (M^+) calcd for $C_{21}H_{18}N_2O_5$ 378.1216, found 378.1215. IR (KBr) 1785, 1771, 1726, 1682 cm^{-1}.

Method B. (±)-2-Phthalimidoglutaric acid **5** (62 mg, 0.224 mmol) was dissolved in dry acetonitrile (2 mL) and cooled to 0 °C, and triethylamine (78 μL, 0.560 mmol) was added to the mixture while stirring. To the resultant white suspension was added 1-hydroxybenzotriazole (123 mg, 0.911 mmol), 4-methoxybenzylamine (35 μL, 0.268 mmol), and 1,3-dicyclohexylcarbodiimide (236 mg, 1.14 mmol). The reaction mixture was allowed to warm to room temperature over 1 h followed by heating at 50 °C for 15 h. The reaction solvent was removed under vacuum, and the yellow residue was dissolved in ethyl acetate and filtered through a short pad of silica gel. Concentration of the filtrate followed by flash-column chromatography (hexane/ethyl acetate, 2:1) provided 46 mg (54%) of **7** by trituration of the combined chromatographic fractions with ethyl acetate. The spectral details were identical to the product prepared above by method A.

1-(4-Methoxybenzyl)-3-(1-oxo-1,3-dihydroisoindol-2-yl)piperidine-2,6-dione (10). Aluminum amalgam was prepared by rinsing coils of food-grade aluminum foil (68 mg, 2.5 mmol) with diethyl ether. Each coil was immersed in 2% aqueous mercuric chloride solution while agitating (20 s), followed by immersing in distilled water (1−2 s) and then adding to a solution of **7** (1.0 mmol) in THF (5 mL) and distilled water (250 μL, 13.9 mmol). The reaction mixture was stirred at room temperature (3 h), during which time evolution of hydrogen occurred with the formation of a gray suspension. The reaction mixture was then vacuum-filtered through Celite while washing with methanol. The clear filtrate was concentrated to a syrup under aspirator vacuum followed by further subjection to high vacuum (12 h) to remove residual water. The syrupy crude lactam **8** was dissolved in dry dichloromethane (3 mL) followed by addition of thiophenol (190 μL, 2.1 mmol) and *p*-toluenesulfonic acid (9.0 mg, 0.05 equiv). The reaction mixture was then stirred under an atmosphere of nitrogen at room temperature (15 h). Upon completion of the reaction as determined by TLC analysis, the reaction mixture was diluted with dichloromethane, washed with 10% aqueous sodium bicarbonate solution, and dried over anhydrous sodium sulfate. Removal of the drying agent followed by concentration and flash-column chromatography (hexane/ethyl acetate, 1:1) of the residue afforded the sensitive phenylthiolactam **9**. The semipure phenylthiolactam **9** was added to 95% ethanol (10 mL) followed by an aqueous suspension of Raney nickel (2 mL). The black suspension was stirred at room temperature (5 h) while monitoring by TLC. After completion of the desulfurization, the reaction mixture was filtered through Celite under gentle suction while washing the filter cake with methanol to prevent the excess Raney nickel from drying. The filtrate was concentrated and flash-column-chromatographed (toluene/ethyl acetate, 2:1) to furnish **10** (291 mg, 80%) as white crystals. Mp 68−71 °C. R_f = 0.36 (hexane/ethyl acetate, 1:1). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.88 (d, J = 7.8 Hz, 1H), 7.51 (dt, J = 0.98 Hz, 7.3, 1H), 7.47 (m, 2H), 7.32 (d, J = 8.8 Hz, 2H), 6.80 (d, J = 8.8 Hz, 2H), 5.20 (dd, J = 5.4, 13.7 Hz, 1H), 4.88 (s, 2H,), 4.42 (d, J = 16.1 Hz, 1H), 4.30 (d, J = 16.1 Hz, 1H), 3.77 (s, 3H), 2.97 (ddd, J = 2.4, 4.9, 18.1 Hz, 1H), 2.85 (ddd, J = 5.4, 13.7, 18.1 Hz, 1H), 2.28 (ddd, J = 4.9, 13.7, 26.3 Hz, 1H), 2.14 (m, J = 2.4, 5.4, 12.7 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 171.2, 170.2, 169.5, 141.7, 132.2*, 131.8, 130.8*, 129.3, 128.4*, 124.3*, 123.2, 114.0*, 55.5*, 52.8*, 47.3, 43.4, 32.4, 22.9. HRMS (FAB) *m/z* ($M + H)^+$ calcd for $C_{21}H_{21}N_2O_4$ 365.1501, found 365.1495. IR (KBr): 1702, 1686, 1613 cm^{-1}.

3-(1-Oxo-1,3-dihydroisoindol-2-yl)piperidine-2,6-dione (1). The *N*-PMB derivative **10** (66 mg, 0.181 mmol) was dissolved in acetonitrile (2 mL). Water (400 μL) was added followed by ceric ammonium nitrate (405 mg, 0.74 mmol). The bright-orange solution was stirred (2 h) and then quenched with aqueous sodium bicarbonate (1:1, w/w) and sodium bisulfite (1:1, w/w) solutions. The volatile components were removed under vacuum, and the solid residue was suspended in acetone followed by filtration through a short silica gel (70−230 mesh) column. Flash-column silica gel was then added to the solution, the solvent was evaporated, and the silica gel with the adsorbed compound was applied to a flash-chromatography column and eluted with dichloromethane/acetone (10:1). Upon elution of all the byproduct 4-methoxybenzaldehyde, the polarity of the solvent system was increased to 2:1. The homogeneous fractions were combined to provide 27 mg (61%) of **1** as a white amorphous solid. Recrystallization of an analytical sample from methyl cellosolve gave **1** as white crystals. Mp 237−238 °C. R_f = 0.22 (dichloromethane/acetone, 5:1). ^{1}H NMR (500 MHz, methanol-d_4) δ: 7.82 (d, J = 7.3 Hz, 1H), 7.65 (t, J = 7.3 Hz, 1H), 7.60 (d, J = 7.3 Hz, 1H), 7.54 (t, J = 7.3 Hz, 1H), 5.17 (dd, J = 5.4 Hz, 13.7, 1H), 4.51 (m, J = 17.0, 27.3 Hz, 2H), 2.92 (ddd, J = 5.4, 13.7, 18.1 Hz, 1H), 2.79 (ddd, J = 2.4, 4.4, 13.7 Hz, 1H), 2.51 (ddd, J = 4.9, 13.2, 26.3 Hz, 1H), 2.08 (m, J = 2.4, 5.4, 18.1 Hz, 1H). ^{13}C NMR (125 MHz, methanol-d_4) δ: 173.5, 171.0, 170.3, 142.5, 132.2*, 131.5, 128.1*, 123.2*, 52.5*, 31.2, 22.9. HRMS *m/z* (M^+) calcd for $C_{13}H_{12}N_2O_3$ 244.0848, found 244.0842. Anal. Calcd for $C_{13}H_{12}N_2O_3$: C, 63.92; H, 4.95; N, 11.45. Found: C, 63.73; H, 4.73; N, 11.05. IR (KBr): 3204, 3099, 1732, 1705, 1680 cm^{-1}.

2-(1-Benzyl-2,6-dioxopiperidin-3-yl)isoindol-1,3-dione (12). *N*-Benzylthalidomide (**12**) was prepared (13%) from (±)-3-phthalimidophthalic anhydride **4** (871 mg, 3.36 mmol) and benzylamine (370 mg, 3.39 mmol) in a manner analogous to that of **7** by method A. R_f = 0.42 (hexane/ethyl acetate, 1:1). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.86 (m, J = 2.9, 5.4 Hz, 2H), 7.75 (m, J = 2.9, 5.4 Hz, 2H), 7.37 (d, J = 8.3 Hz, 2H), 7.29 (t, J = 7.3 Hz, 2H), 7.23 (t, J = 7.3 Hz, 1H), 5.08 (d, J = 14.2 Hz, 1H), 5.03 (m, 1H), 4.91 (d, J = 14.2 Hz, 1H), 2.97 (dt, J = 29, 15.6 Hz, 1H), 2.80 (m, 2H), 2.10 (m, 1H). ^{13}C NMR (125 Mz, $CDCl_3$) δ: 170.1, 168.9, 167.6, 136. 8, 134.6*, 132.0, 128.9*, 128.7*, 127.8*, 124.0*, 50.5*, 44.1, 32.3, 22.2. HRMS *m/z* (M^+) calcd for $C_{20}H_{16}N_2O_4$ 348.1110, found 348.1121. IR (KBr): 1772, 1724, 1687, 1610 cm^{-1}.

1-Benzyl-3-(1-oxo-1,3-dihydroisoindol-2-yl)piperidine-2,6-dione (13). The *N*-benzyl derivative (**13**) was prepared in the same manner as *N*-PMB derivative (**10**) starting from 2-(1-benzyl-2,6-dioxopiperidin-3-yl)isoindol-1,3-dione (*N*-benzylthalidomide) **12** (102 mg, 0.29 mmol). Flash column chromatography (toluene/ethyl acetate, 2:1) provided 73 mg (75%) of **13** as a white amorphous solid. R_f = 0.15 (hexane/EtOAc, 1:1). ^{1}H NMR ($CDCl_3$) δ: 7.89 (d, J = 7.8 Hz, 1H), 7.57 (t, J = 7.8 Hz, 1H), 7.47 (m, 2H), 7.37 (d, J = 7.8 Hz, 2H), 7.27 (m, 3H), 5.28 (dd, J = 5.4, 13.7 Hz, 1H), 4.96 (s, 2H), 4.43 (d, J = 15.6 Hz, 1H), 4.32 (d, J = 15.6 Hz, 1H), 2.99 (m, 1H), 2.87 (ddd, J = 5.4, 13.7, 17.6 Hz, 1H), 2.31 (ddd, J = 4.4, 13.2, 26.3 Hz, 1H), 2.16 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 171.2, 170.2, 169.5, 141.7, 137.0, 132.2*, 131.8, 129.2*, 128.7*, 128.4*, 127.9*, 124.4*, 123.2*, 52.7*, 47.4, 44.0, 32.4, 23.0. HRMS *m/z* (M^+) calcd for $C_{20}H_{18}N_2O_3$ 334.1317, found 334.1322. IR (KBr): 1770, 1721, 1708, 1662, 1621 cm^{-1}.

2-(1-Benzyloxy-2,6-dioxopiperidin-3-yl)isoindol-1,3-dione (14). (±)-2-Phthalimidoglutaric acid **5** (5.022 g, 18.1 mmol) was heated with acetic anhydride (25 mL, mmol) and trifluoroacetic anhydride (0.5 mL, mmol) under an atmosphere of nitrogen at 100° C for 13 h. The volatile components were removed under high vacuum, which left a white residue of crude anhydride **4**. Pyridine (15 mL, mmol) was added, and then benzyloxyamine hydrochloride (2.89 g, 18.1 mmol) was added. The mixture was then heated under an atmosphere of nitrogen at 60 °C (3 h) followed by removal of the pyridine under high vacuum at room temperature to yield a dark syrupy residue. To the residue was added acetic anhydride (25 mL, mmol) and trifluoroacetic anhydride (0.5 mL, mmol) followed

by heating at 100 °C (4 h) under an atmosphere of nitrogen. Concentration of the reaction mixture under high vacuum followed by flash-column chromatography (hexane/ethyl acetate, 2:1) afforded *N*-benzyloxythalidomide **14** (6.38 g, 97%) as a white foam. $R_f = 0.32$ (hexane/ethyl acetate, 1:1). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.90 (m, $J = 2.9$, 5.4 Hz, 2H), 7.78 (m, $J = 2.9$, 5.4 Hz, 2H), 7.55 (m, 2H), 7.37 (m, 3H), 5.06 (s, 2H), 5.09−5.04 (m, 1H), 3.00−2.9 (m, 1H), 2.83−2.74 (m, 2H), 2.16−2.08 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 167.4, 167.1, 165.3, 134.8*, 134.0, 131.9, 130.3*, 129.4*, 128.7*, 124.1*, 78.7, 50.6*, 31.5, 22.2. HRMS *m/z* $(M+H)^+$ calcd for $C_{20}H_{17}N_2O_5$ 365.1137, found 365.1123. IR (KBr): 1784, 1771, 1751, 1719 cm^{-1}.

1-Benzyloxy-3-(1-oxo-1,3-dihydroisoindol-2-yl)piperidine-2,6-dione (15). *N*-Benzyloxythalidomide **14** (634 mg, 1.74 mmol) was dissolved in THF (6.0 mL) followed by the addition of water (0.5 mL, 27.7 mmol). To the vigorously stirred solution was added aluminum amalgam, which was prepared from aluminum foil (141 mg, 5.22 mmol) as described above. After the reduction was complete (3 h), the resulting gray suspension was diluted with methanol (10 mL) and vacuum-filtered through Celite. The clear filtrate was concentrated under reduced pressure, and the residue was dissolved in dry dichloromethane (10 mL). The solution was cooled to 0 °C. Then trifluoroacetic acid (270 μL, 3.5 mmol) was added, and the resulting yellow solution was stirred for 10 min. Triethylsilane (834 μL, 5.22 mmol) was then slowly added, and stirring was continued for 2 h at 0 °C. The reaction mixture was then concentrated under vacuum and purified by flash chromatography (toluene/ethyl acetate, 3:1) to provide **15** (251 mg, 41%) as a white amorphous solid. $R_f = 0.23$ (hexane/ethyl acetate, 1:1). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.88 (d, $J = 7.8$ Hz, 1H), 7.58 (t, $J = 7.8$ Hz, 1H), 7.50 (m, 3H), 7.44 (d, $J = 7.8$ Hz, 1H), 7.37 (m, 3H), 5.25 (m, 1H), 5.04 (dq, $J = 2.4$, 9.3 Hz, 2H), 4.37−4.22 (dd, 2H), 2.94 (m, 1H), 2.84 (m, 1H), 2.24 (m, 1H), 2.10 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 169.4, 167.5, 166.7, 141.7, 133.9, 132.3*, 131.6, 130.4*, 129.5*, 128.7*, 128.5*, 123.2*, 78.4, 53.1*, 53.0*, 47.3, 31.6, 22.9. HRMS *m/z* $(M + H)^+$ calcd for $C_{20}H_{18}N_2O_4$ 351.1346, found 351.1340. IR (KBr): 1773, 1716, 1699, 1600, 1621 cm^{-1}.

Preparation of 1 from the *N*-Benzyloxy Derivative (15). *N*-Benzyloxy-protected **15** (4.69 g, 13.4 mmol)) was dissolved in methanol (30 mL). Palladium on activated carbon (10%, 354 mg) was carefully added, and the mixture was stirred under an atmosphere of hydrogen (1 atm, 24 h). The catalyst was removed by filtration through Celite, and the filtrate was concentrated under reduced pressure. Anhydrous acetonitrile (150 mL) was then added to the syrupy residue followed by the addition of α-bromoacetophenone (2.66 g, 13.4 mmol), triethylamine (1.9 mL, 13.4 mmol), and 4-(dimethylamino)pyridine (166 mg, 1.36 mmol). The resulting yellow solution was stirred (4 h) and was then concentrated under vacuum. The dark-orange syrupy resudue was flash-chromatographed twice (toluene/ethyl acetate, 5:1; then toluene/ethyl acetate, 3:1) to afford 700 mg (21%) of **1** as white crystals. The compound exhibited the same melting point and spectral characteristics as those of the compound obtained from the N-deprotection of the *N*-PMB derivative (**10**).

Preparation of 1 by Direct Reduction−Deoxygenation of (±)-Thalidomide (2). A suspension of racemic thalidomide **2** (4.52 g, 1.75 mmol) in ethanol (100 mL) was heated to reflux (5 min). Water (10 mL) waas added followed by glacial acetic acid (5 mL). Aluminum foil (1.41 g, 5.26 mmol) was cut into 1 cm^2 pieces, rinsed with diethyl ether, and added to the thalidomide/acetic acid/water suspension. To the well-stirred suspension, mercury(II) chloride (22 mg, 81 μmol) was added, and the mixture was then refluxed.

During the reaction, aliquots were analyzed by NMR, which indicated that complete conversion to the corresponding hydroxylactam was complete in 3 h. The reaction mixture was cooled, vacuum-filtered through a thin pad of Celite, and concentrated under vacuum to give the crude hydroxylactam as a white amorphous solid. The crude hydroxylactam was dried under vacuum at room temperature and then mixed with glacial acetic acid (15 mL). Trifluoroacetic acid (9 mL, 121 mmol) was then added, and the mixture was heated at 70 °C (10 min). Triethylsilane (6.6 mL, 41.3 mmol) was slowly added, and heating was continued (3 h) at 70 °C. The resulting heterogeneous mixture was allowed to cool to room temperature, diluted with methanol (50 mL), and then vacuum-filtered through Celite. The pale-yellow filtrate was concentrated to a solid and then traces of acetic and trifluoroacetic acid were removed under vacuum (0.5 Torr) at 60 °C (14 h). The solid residue was triturated with hot ethyl actetate and then cooled to −10 °C for 2 h. The solid precipitate (2.41 g) was collected by filtration, washed twice with ethyl acetate (40 mL), and dried under vacuum. The filtrate was reduced to 10 mL and provided another 0.96 g of crude product that was collected by filtration. Combining both crops of crude product and recrystallization from methyl cellosolve afforded 2.83 g (66%) of pure **1** with melting points and spectral data that are identical to those of the samples prepared above.

Rat Aortic Ring Culture Bioassay. Female 6−8 week old Sprague−Dawley rats were euthanized by asphyxiation with CO_2 followed by removal of the thoracic aorta. With a dissecting microscope, 1 mm thick rings were then cut. Each ring was rinsed eight times with culture medium, embedded in Matrigel (Collaborative Biomedical, Bedford, MA), and incubated in growth-factor-free endothelial cell basal medium (EBM). The cell basal medium (Clonetics, Walkersville, MD) contained 10 μg/mL gentiamycin, 100 units/mL penicillin, 100 mg/mL streptomycin, and 0.25 μg/mL amphotericin. At 24 h intervals, the supernatant was replaced with 9 μg/mL **1** dissolved in DMSO. Incubation of the cultures was at 37 °C with 5% CO_2. The experiments utilized aortic rings taken from four different rats. The aortic ring preparations were cultured for 4 days, during which time microvessel growth was assessed daily. To confirm the presence of endothelial cells in the new microvessel growth, they were stained with factor VIII and CD34 by previously detailed methods.[13] Control cultures consisted of EBM/0.05% DMSO and a positive control, carboxyamidotriazole (CAI, 30 μM) in EBM/0.05% DMSO. Digital photographic images of both the control and test cultures were recorded each day. The images of the rat aortic rings (Figure 2) were then scanned onto a photo CD, and the pixel density in each image was quantified by employing a gray-scale analysis of background versus light pixels (outgrowths) as determined by the NIH Image program.[24] The bar graphs in Figure 1, which show relative percent microvessel growth, are derived from the relative pixel density ($(pixel)^2$ units) of the images. The bar graphs were generated using KaleidaGraph software. Comparisons were made using Student's *t*-test with $P < 0.05$ as the criterion for statistical significance. The data were represented as the mean ± SEM ($n = 4$).

Acknowledgment. The research was supported by the Intramural Research Program of the National Cancer Institute.

References

(1) (a) Thalidomide Analogs and Metabolites, Part 3. For Part 2, see the following. Luzzio, F. A.; Thomas, E. M.; Figg, W. D. *Tetrahedron Lett.* **2000**, *41*, 7151−7155. (b) Presented at the 222nd National Meeting of the American Chemical Society, Chicago, IL, August 29, 2001; Paper MEDI 0264.

(2) Rosen, L. Antiangiogenic Strategies and Agents in Clinical Trials. *Oncologist* **2000**, *5* (Suppl.), 20−27.

(3) Helm, F. C.; Frankus, E.; Friderichs, E.; Graudums, I.; Flohé, L. Comparative Teratological Investigation of Compounds Structurally and Pharmacologically Related to Thalidomide *Arzneim.-Forsch.* **1981**, *31*, 941−949.

(4) (a) Udagawa, T.; Verheul, H. M. W.; D'Amato, R. J. Thalidomide and Analogs. In *Antiangiogenic Agents in Cancer Therapy*; Teicher, B. A., Ed.; Humana Press: Totowa, NJ, 1999; pp 263−274. (b) Figg, W. D.; Reed, E.; Green, S.; Pluda, J. Thalidomide A Prodrug That Inhibits Angiogenesis. In *Antiangiogenic Agents in Cancer Therapy*; Teicher, B. A., Ed.; Humana Press: Totowa, NJ, 1999; pp 407−422.

(5) Schmahl, H.-J.; Nau, H.; Neubert, D. The Enantiomers of the Teratogenic Thalidomide Analogue EM 12. *Arch. Toxicol.* **1988**, *62*, 200−204.

(6) Heger, W.; Klug, S.; Schmahl, H.-J.; Nau, H.; Merker, H.-J.; Neubert, D. Embryotoxic Effects of Thalidomide Derivatives on the Non-Human Primate *Callithrix jacchus*. *Arch. Toxicol.* **1988**, *62*, 205−208.
(7) Schmahl, H.-J.; Heger, W.; Nau, H. The Enantiomers of the Teratogenic Thalidomide Analogue EM 12. *Toxicol. Lett.* **1989**, *45*, 23−33.
(8) Winckler, K.; Klinkmüller, K. D.; Schmal, H. J. Determination of the Thalidomide Analogues 2-(2,6-Dioxopiperidine-3-yl) Phthalimidine (EM 12), 2-(2,6-dioxopiperidine-4yl) Phthalimidine (EM 16) and Their Metabolites in Biological Samples. *J. Chromatog. Biomed. Appl.* **1989**, *488*, 417−425.
(9) Kaplan, G.; Sampaio, E. P. Method of Treating Abnormal Concentrations of TNFα U.S. Patent 5,385,901, 1992 (to Rockefeller University); *Chem. Abstr.* **1992**, *117*, 226313a.
(10) Shah, J. H.; Swartz, G. M.; Papathanassiu, A. E.; Treston, A. M.; Fogler, W. E.; Madsen, J. W.; Green, S. J. Synthesis and Enantiomeric Separation of 2-Phthalimidinoglutaric Acid Analogues: Potent Inhibitors of Tumor Metastasis. *J. Med. Chem.* **1999**, *42*, 3014−3017.
(11) Neubert, R.; Nogueira, A. C.; Neubert, D. Thalidomide Derivatives and the Immune System I. Changes in the Pattern of Integrin Receptors and Other Surface Markers on T Lymphocyte Subpopulations of Marmoset Blood. *Arch. Toxicol.* **1993**, *67*, 1−17.
(12) D'Amato, R. J.; Loughnan, M. S.; Flynn, E.; Folkman, J. Thalidomide is an Inhibitor of Angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4082−4085.
(13) Bauer, K. S.; Dixon, S. C.; Figg, W. D. Inhibition of Angiogenesis by Thalidomide Requires Metabolic Activation Which Is Species-Dependent. *Biochem. Pharmacol.* **1998**, *55*, 1827−1834.
(14) Hansen, J. M.; Carney, E. W.; Harris, C. Differential Alteration by Thalidomide of the Glutathione Content of Rat vs. Rabbit Conceptuses in Vitro. *Reprod. Toxicol.* **1999**, *13*, 547−554.
(15) (a) Green, T. W.; Wuts, P. G. M. *Protective Groups in Organic Synthesis*, 2nd ed.; Wiley: New York, 1991; p 400. (b) Although the PMB route to **1** was described in an earlier publication (see ref 17), no experimental details were provided. Full experimental details are provided in this report where the yields in some steps differ because of scale-up.
(16) Luzzio, F. A.; Mayorov, A. V.; Figg, W. D. Thalidomide Metabolites. Part I: Derivatives of (+)-2-*N*-Phthalimido-γ-hydroxyglutamic Acid *Tetrahedron Lett.* **2000**, *41*, 2275−228.
(17) Luzzio, F. A.; Piatt Zacherl, D. P.; Figg, W. D. A Facile Scheme for Phthalimide/-Phthalimidine Conversion. *Tetrahedron Lett.* **1999**, *40*, 2087−2090.
(18) Guo, Z.; Schultz, A. G. Organic Synthesis Methodology. Preparation and Diastereoselective Birch Reduction−Alkylation of 3-Substituted 2-Methyl-2,3-Dihydroisoindol-1-ones. *J. Org. Chem.* **2001**, *66*, 2154−2157.
(19) (a) Benzylamines are not well-suited for glutarimide formation because of their lower nucleophilicity (see ref 20). In contrast, β-phenethylamine gave the corresponding glutarimide in 83% yield using method A. (b) Fox, D. J.; Reckless, J.; Warren, S. J.; Grainger, D. J. Design, Synthesis and Preliminary Pharmacological Evaluation of *N*-Acyl-3-aminoglutarimides as Broad-Spectrum Chemokine Inhibitors in Vitro and Anti-inflammatory Agents in Vivo. *J. Med Chem.* **2002**, *45*, 360−370.
(20) Robin, S.; Zhu, J.; Galons, H.; Pham-Huy, C.; Claude, J. R.; Tomas, A.; Viossat, B. A Convenient Asymmetric Synthesis of Thalidomide. *Tetrahedron: Asymmetry* **1995**, *6*, 1249−1252. The electron-donating capacity of the NBO group presumably reduces racemization of the product thalidomide.
(21) Hart, D. J.; Yang, T.-K. *N*-Acyliminium Ion Rearrangements: Generalities and Application to the Synthesis of Pyrrolizidine Alkaloids. *J. Org. Chem.* **1985**, *50*, 235−242.
(22) Nicosia, R. F.; Ottinetti, A. Growth of Microvessels in Serum-Free Matrix Culture of Rat Aorta. *Lab. Invest.* **1990**, *63*, 115−122.
(23) Hamby, J. M.; Showalter, H. D. H. Small Molecule Inhibitors of Tumor-Promoted Angiogenesis, Including Protein Tyrosine Kinase Inhibitors. *Pharmacol. Ther.* **1999**, *82*, 169−193.
(24) The NIH Image program was developed at the U.S. National Institutes of Health and is available on the Internet at http://rsb.info.nih.gov/nih-image/.

JM020079D