Mono- and Dihydroxylated Metabolites of Thalidomide: Synthesis and TNF-α Production-Inhibitory Activity

Takanori NAKAMURA, Tomomi NOGUCHI, Hisayoshi KOBAYASHI, Hiroyuki MIYACHI, and Yuichi HASHIMOTO*

Institute of Molecular & Cellular Biosciences, The University of Tokyo; 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–0032, Japan.
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Mono- and dihydroxylated metabolites of thalidomide were efficiently prepared and characterized, and their inhibitory activity on tumor necrosis factor (TNF)-α production in the human monocytic leukemia cell line THP-1 was evaluated. 5,*N*-Dihydroxythalidomide was a much more potent TNF-α production inhibitor than thalidomide.

Key words thalidomide; hydroxythalidomide; metabolite; tumor necrosis factor (TNF)-α modulator

Thalidomide (**1**) was developed in the 1950's as a nontoxic sedative/hypnotic drug, but was withdrawn from the market in the early 1960's because of its serious teratogenicity.[1—5] However, it was subsequently identified as an effective agent for the treatment of multiple myeloma (MM), AIDS, Hansen's disease, and various cancers.[1—5] The US Food and Drug Administration (FDA) approved it for the treatment of erythema nodosum in Hansen's disease in 1998, and (in combination with dexamethasone) for the treatment of MM in 2006. Official approval for the use of thalidomide (**1**) to treat MM has also been applied for in Japan. Thalidomide (**1**) has been discovered to have various biological activities, including inhibition of tumor necrosis factor-α (TNF-α) production, and anti-inflammatory, anti-angiogenic, and cyclooxygenase (COX)-inhibitory activities.[1—5] The TNF-α production-inhibitory activity was initially considered to be one of the key mechanisms of thalidomide's actions,[1—5] though the precise molecular mechanism(s) involved remain unclear.

Recently, we have reported that thalidomide (**1**) and/or its two hydroxylated metabolites 5-OH-Thal (**2a**) and *N*-OH-Thal (**2c**) show cell differentiation-enhancing, anti-angiogenic and tubulin polymerization-inhibitory activities.[6—9] Concerning the former two activities, thalidomide (**1**), 5-OH-Thal (**2a**) and *N*-OH-Thal (**2c**) all exhibit comparable activity.[7—9] However, tubulin polymerization-inhibitory activity was observed only for the hydroxylated metabolites (**2a**, **2c**), and thalidomide (**1**) lacks this activity.[6] These results prompted us to investigate comprehensively the biological activities of hydroxylated metabolites of thalidomide.

Thalidomide (**1**) is metabolically labile, and many metabolites have been identified or proposed,[10—17] including seven hydroxylated metabolites, **2a**—**g** (Fig. 1). Hydroxylation is reported to occur mainly at the 5-position in the phthaloyl moiety and the 5′-position in the glutarimide moiety,[10] although the 4-position of the phthaloyl moiety and the nitrogen atom of the imide ring can also be hydroxylated (Fig. 1).[10] 5-OH-Thal (**2a**), *N*-OH-Thal (**2c**)[11—13] and *cis*-5′-OH-Thal (*cis*-**2f**)[14—16] have been well-characterized and their methods of preparation have been reported in detail. For 4-OH-Thal (**2b**), *trans*-5′-OH-Thal (*trans*-**2f**), and the dihydroxylated metabolites, 5,*N*-di-OH-Thal (**2d**), 4,*N*-di-OH-Thal (**2e**), and 5,5′-di-OH-Thal (**2g**), neither spectroscopic data nor any detailed description of their synthesis is available in the literature, to our knowledge. In addition, the reported synthetic method of *cis*-5′-OH-Thal (*cis*-**2f**) *via* free γ-hydroxyglutamic acid is unsatisfactory, because some of the intermediates are sticky and intractable, the overall yield is not so high, and the stereochemistry is difficult to control. We therefore sought to establish methods for systematic preparation of all of the proposed metabolites, **2a**—**g**. In this paper, we describe the synthesis of the mono- and dihydroxylated metabolites of thalidomide (**2a**—**g**), as well as the results of chemical/physical characterization and evaluation of their TNF-α production-inhibitory activity.

Chemistry

Hydroxylated metabolites **2a**—**e** were synthesized as shown in Chart 1.

Boc-glutamine **3** was treated with 1,1′-carbonyldiimidazole (CDI) in the presence of a catalytic amount of 4-dimethylaminopyridine (DMAP) to afford the cyclic imide **4**.[18] The Boc group of **4** was removed with 30% HBr/AcOH to afford **5** as the HBr salt. Boc-glutamic acid **6** was treated with *O*-benzylhydroxylamine in the presence of *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDCI) and 1-hydroxybenzotriazole (HOBt),[13] to afford the cyclic imide **7**. The Boc group was removed with trifluoroacetic acid (TFA) to afford **8** as the TFA salt. The hydroxyl groups of 4-hydroxyphthalic acid (**9a**) and 3-hydroxyphthalic acid (**9b**) were protected as the benzyl ether, and the protected compounds were

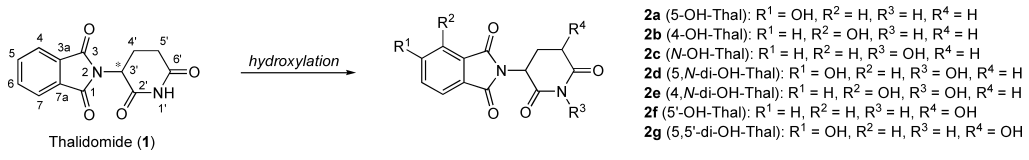
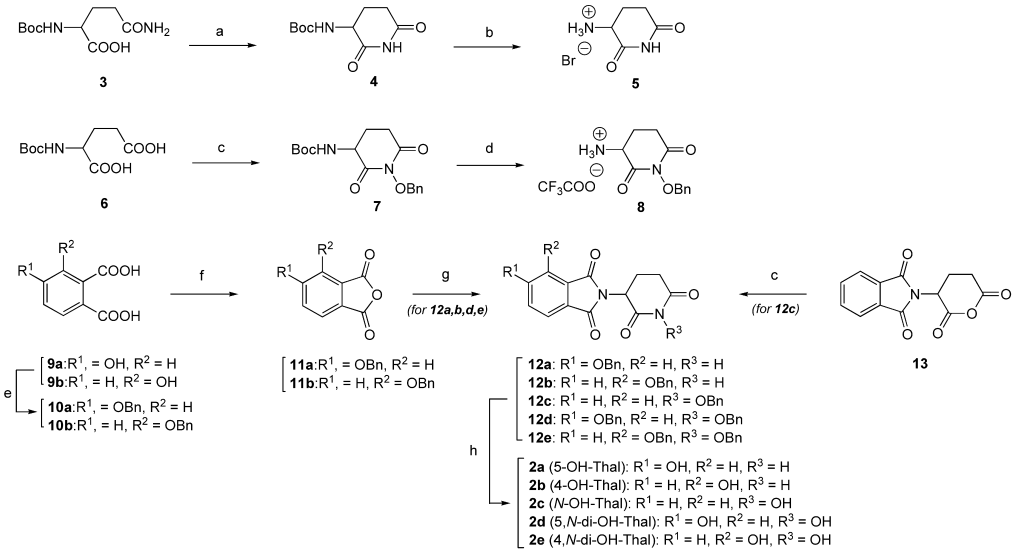


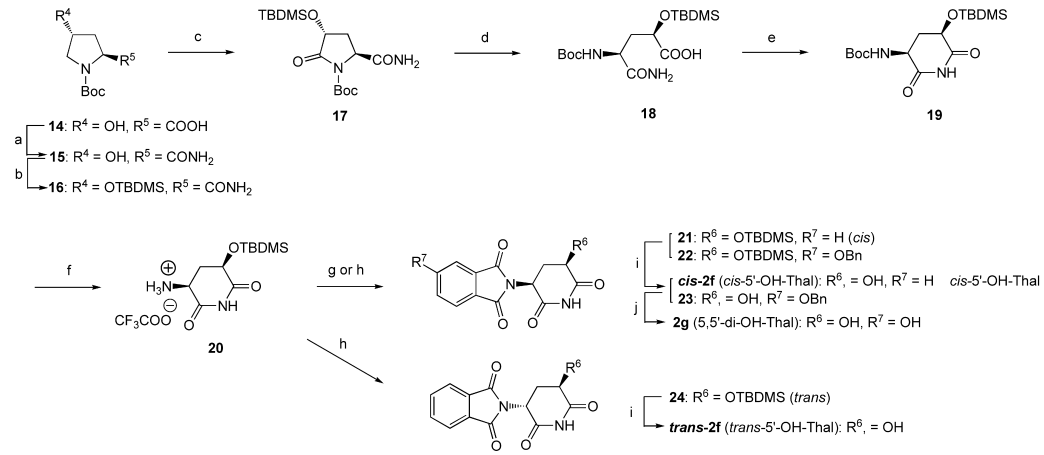
Fig. 1. Structures of Thalidomide (**1**) and Its Hydroxylated Metabolites (**2**)

* To whom correspondence should be addressed. e-mail: hashimot@iam.u-tokyo.ac.jp



Reagents and conditions: (a) CDI, DMAP, TEA, THF, rt, 3 days (90%) ; (b) 30% HBr /AcOH, rt, 30 min (98%) ; (c) NH_2-OBn· HCl, EDCI, HOBt, TEA, CH_2Cl_2, rt, 1–3 days (84–87%) ; (d) TFA, rt, 30 min (quant.) ; (e) BnCl, KOH, H_2O, reflux, 10–12 h (77%–quant.) ; (f) Δ, 200ºC, neat, 1 h (60–80%) ; (g) **5** or **8**, EDCI, HOBt, TEA, CH_2Cl_2, rt, 3 days (30–98%) ; (h) H_2, 10% Pd-C, AcOH/AcOEt or MeOH/DMF, rt, 1–4 h (59–88%).

Chart 1



Reagents and conditions: (a) EDCI, HOBt, CH_3CN, then NH_3 (28% aq.), 0ºC to rt, 7 h (100%) ; (b) TBDMS-Cl, Imidazole, THF, rt, 1 day (87%) ; (c) $NaIO_4$, RuO_2·nH_2O, AcOEt/H_2O, rt, 3 days (85%) ; (d) LiOH (1 mol/L aq.), THF, 0ºC, 3 h ; (e) CDI, DMAP, DIPEA, CH_2Cl_2, rt, 5 days (33%, 2 steps); (f) TFA, CH_2Cl_2, rt, 30 min (quant.); (g) Phthalic anhydride, EDCI, HOBt, DIPEA, CH_2Cl_2, 0ºC, 30 min (54% 2 steps) ; (h) Phthalic anhydride or **11a**, EDCI, HOBt, TEA, CH_2Cl_2, rt, 1–2 days (25–44% 2 steps) ; (i) TBAF, THF, 0ºC, 30–90 min (28–86%) (j) H_2, 10% Pd-C, 1,4-Dioxane, rt, 5 h (98%).

Chart 2

heated to 200 °C for 2 h without solvent to give the corresponding anhydrides **11a** and **11b**, respectively. These compounds were treated with **5** or **8** in the presence of EDCI and HOBt to afford **12a**—**e**, except **12c** which was prepared from **13**. The benzyl group of **12a**—**e** was removed by catalytic hydrogenation with H_2 gas on 10% Pd/C to afford the hydroxylated thalidomide derivatives **2a**—**e**.

5′-Hydroxylated metabolites [5′-OH-Thal (**2f**) and 5′,*N*-di-OH-Thal (**2g**)] were synthesized as shown in Chart 2.

Trans-*N*-Boc-L-hydroxyproline **14** was treated with aqueous ammonia (28%) in the presence of EDCI and HOBt as coupling reagents to give the carboxamide **15**.[19] Protection of the hydroxyl group of **15** as the *tert*-butyldimethylsilyl (TBDMS) ether **16**, followed by oxidation with $NaIO_4$ and a catalytic amount of RuO_2 in AcOEt/H_2O at room temperature afforded the pyroglutamic amide derivative **17**.[20] Interestingly, $NaIO_4$/RuO_2 oxidation progressed only in the case of **16** as a substrate (neither the *N*-Cbz protected derivative of **16** nor the *O*-Bn protected derivative of **16** could be oxidized under the same conditions to afford the corresponding pyroglutamic amide derivatives of **17**). The lactam part of **17** was hydrolyzed with 1 mol/l LiOH in THF at 0 °C to afford the γ-hydroxyglutamine derivative **18**,[21,22] but the desilylated product was formed as a by-product. Although several kinds of alkaline hydrolysis were tried, *e.g.*, with KOH and NaOH, the by-product was always formed to some extent. Compound **18** was cyclized using CDI in the presence of a catalytic amount of DMAP to afford the glutarimide derivative **19**. The Boc group was selectively removed in the presence of TBDMS ether with TFA/CH_2Cl_2 to afford **20** as the TFA salt.[23] Careful reaction of **20** and phthalic anhydride in the presence of EDCI and HOBt at 0 °C for 30 min afforded only the *cis*-

isomer **21** (epimerization may occur at this step). When the reaction time was extended to 2 d at room temperature, epimerization occurred to afford a *ca.* 1 : 1 mixture of *cis*- and *trans*-isomers. *Trans*-5′-OTBDMS-thalidomide **24** was isolated by recrystallization. Interestingly, *cis*-5′-acetoxythalidomide was reported to be more crystallizable than *trans*-5′-acetoxythalidomide.[14] Therefore, the crystallizability may depend on the nature of the 5′-hydroxyl substituent. Finally, removal of the TBDMS group of **21** with tetra-*n*-butylammonium fluoride (TBAF) at 0 °C gave the desired *cis*-5′-OH-Thal (*cis*-**2f**) stereoselectively. *Trans*-5′-OH-Thal (*trans*-**2f**) was also prepared by means of a procedure similar to that used for *cis*-5′-OH-Thal (*cis*-**2f**). Although it was not clear whether the 3′ or 5′ position was epimerized, probably the two were not epimerized simultaneously, judging from the large optical rotation values (see Experimental). This synthetic route is economical, and allows stereo-controlled preparation of *cis*-5′-OH-Thal (*cis*-**2f**). 5′-OH-Thal's (*cis*- and *trans*-**2f**) thus obtained were optically unstable, *i.e.*, this compound epimerizes rapidly to a 1 : 1 mixture of *cis*- and *trans*-isomers under heating at 100 °C (data not shown).

TNF-α Production-Inhibitory Activity TNF-α is one of the cytokines mediating immune regulation, and has a wide range of activities extending beyond the well-characterized pleiotropic pro-inflammatory properties to include diverse signaling for cell differentiation, proliferation and death.[24,25] The growing understanding of the pathophysiological role of TNF-α in various diseases, including tumors, as well as the discovery of the TNF-α production-inhibitory activity of thalidomide (**1**),[26] has led researchers to regard this activity as the molecular basis for the pharmacological effects elicited by thalidomide (**1**).[27] Although thalidomide (**1**) has recently been found to be a multi-target drug,[1—5] TNF-α production-regulating activity is still regarded as one of the major molecular mechanisms of thalidomide's action,[1] and the drug is recognized as an immunomodulatory agent. Because thalidomide (**1**) is a metabolically labile compound, as mentioned before, the question of whether its metabolites retain this activity or not is of great interest.

For this reason, we investigated the TNF-α production-inhibitory activity of the hydroxylated metabolites of thalidomide (**2a**—**g**) by means of the previously reported method, using a human monocytic leukemia cell line, THP-1.[28,29] THP-1 cells do not produce TNF-α under normal cell culture conditions, but do produce TNF-α in response to 12-*O*-tetradecanoylphorbol 13-acetate (TPA).[30,31] Thalidomide (**1**) shows moderate TNF-α production-inhibitory activity in this assay system.[1—5,28,29] The TNF-α production-inhibitory activity of test compounds was measured as described in the experimental section as %-inhibition values, *i.e.*, 100% and 0% represent complete inhibition and no inhibition, respectively. Of course the %-values were variable from experiment to experiment, but the order of efficacy was reproducible. A typical set of data collected at the test compound concentration of 30 μM is presented in Table 1.

The mother compound, thalidomide (**1**) showed moderate TNF-α production-inhibiting activity (64.1% inhibition at 30 μM), in accordance with our previous reports.[28,29] Mono-hydroxylation at the phthalimide/aromatic moiety [5-OH-Thal (**2a**) and 4-OH-Thal (**2b**)] caused loss of the activity (−2.1—6.2%). Mono-hydroxylation at the nitrogen atom of the glutarimide ring [*N*-OH-Thal (**2c**)] decreased the activity, though the compound showed weak TNF-α production-inhibitory activity (29.7% inhibition). On the other hand, mono-hydroxylation at the 5′-position of the glutarimide moiety [5′-OH-Thal (*cis*-**2f** and *trans*-**2f**)] slightly enhanced the activity (72.1—74.0% inhibition). The *cis*- and *trans*-isomers (*cis*-**2f** and *trans*-**2f**) showed almost the same efficacy, possibly because of easy isomerization (*vide supra*).

Table 1. TNF-α Production-Inhibitory Activity of Thalidomide (**1**) and Its Hydroxylated Metabolites (**2a**—**g**) at 30 μM

Compound	TNF-α production-inhibitory activity (%)
Thalidomide (**1**)	64.1
5-OH-Thal (**2a**)	6.2
4-OH-Thal (**2b**)	−2.1
N-OH-Thal (**2c**)	29.7
5,*N*-di-OH-Thal (**2d**)	90.9
4,*N*-di-OH-Thal (**2e**)	69.8
cis-5′-OH-Thal (*cis*-**2f**)	74.0
trans-5′-OH-Thal (*trans*-**2f**)	72.1
5,5′-di-OH-Thal (**2g**)	53.1

Interestingly, although both mono-hydroxylation at the phthalimide/aromatic moiety [5-OH-Thal (**2a**) and 4-OH-Thal (**2b**)] and at the nitrogen atom in the glutarimide moiety [*N*-OH-Thal (**2c**)] caused loss or decrease of the activity, double hydroxylation at these sites [5,*N*-di-OH-Thal (**2d**) and 4,*N*-di-OH-Thal (**2e**)] retained or enhanced the activity (69.8—90.9% inhibition). 5,*N*-di-OH-Thal (**2d**) showed the most potent activity among the compounds examined. It was far more potent than thalidomide (**1**), with an IC_{50} value of 3.7 μM in our assay system (TNF-α production inhibition was almost complete at 30 μM, as shown in Table 1). Another di-hydroxylated analog, 5,5′-di-OH-Thal (**2g**), showed moderate TNF-α production-inhibitory activity (53.1% inhibition), which is weaker than that of thalidomide (**1**) or *cis*- and *trans*-5′-OH-Thal (*cis*- and *trans*-**2f**), but more potent than that of 5-OH-Thal (**2a**).

These results indicate that metabolic hydroxylation of thalidomide (**1**) can result in loss, retention, decrease or increase of TNF-α production-inhibitory activity. Therefore, it is not easy to predict the influence of the metabolism of thalidomide (**1**) on the TNF-α production-inhibitory activity in particular *in vivo* situations. Further studies to investigate the influence of metabolism on other biological activities of thalidomide are under way.

Experimental

General ^{1}H-NMR spectra were obtained on a JEOL JNM-α 500 spectrometer (500 MHz). Mass spectra were obtained on a JEOL JMA-HX 110 spectrometer with *m*-nitrobenzyl alcohol. Melting points, determined on a Yanaco MP-J3 micro melting point apparatus are uncorrected. Optical rotation was measured by JASCO DIP-1000. Flash column chromatographies were performed on silica gel 60 Kanto Kagaku (40—100 μm).

***tert*-Butyl 2,6-Dioxopiperidin-3-ylcarbamate (4)** To a solution of Boc-Gln-OH **3** (10.0 g, 40.6 mmol) in dry THF (100 ml) was added 1,1′-carbonyldiimidazole (CDI) (7.78 g, 48.0 mmol), 4-dimethylaminopyridine (DMAP) (1.22 g, 10.0 mmol) and triethylamine (TEA) (4.86 g, 48.0 mmol). The mixture was stirred at room temperature for 3 d, then H_2O was added. The mixture was extracted with $CHCl_3$ 3 times, and the organic solution was washed with 0.2 mol/l aqueous HCl, and brine, dried over $MgSO_4$, filtered and concentrated. The residue was purified by recrystallization ($CHCl_3$/isopropyl ether) to afford **4** (8.30 g, 36.4 mmol, 90%) as white crystals. ^{1}H-NMR ($CDCl_3$): δ: 8.22 (s, 1H), 5.36 (s, 1H), 4.31 (m, 1H), 2.79 (m, 1H),

2.68 (m, 1H), 2.52 (m, 1H), 1.86 (m, 1H), 1.46 (s, 9H). MS (FAB): *m/z* 229 $(M+H)^+$.

3-Aminopiperidine-2,6-dione Hydrobromide (5) A mixture of **4** (200 mg, 0.877 mmol) and 30% HBr/AcOH (2 ml) was stirred at room temperature for 30 min, then AcOEt was added until a precipitate formed. The precipitate was collected by filtration and washed with AcOEt twice to afford **5** (180 mg, 0.861 mmol, 98%) as a white solid. ^{1}H-NMR (DMSO-d_6): δ: 11.27 (s, 1H), 8.38 (br, 3H), 4.21 (dd, J=13.2, 5.1 Hz, 1H), 2.71 (m, 1H), 2.58 (m, 1H), 2.13 (m, 1H), 1.99 (m, 1H). MS (FAB): *m/z* 129 $(M+H)^+$.

***tert*-Butyl 1-Benzyloxy-2,6-dioxopiperidin-3-ylcarbamate (7)** To a solution of Boc-Glu-OH **6** (3.71 g, 15.0 mmol) in dry CH_2Cl_2 (100 ml) was added *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI·HCl) (11.5 g, 60.0 mmol), 1-hydroxybenzotriazole monohydrate (HOBt·H_2O) (8.11 g, 60.0 mmol), and TEA (9.11 g, 90.0 mmol). The reaction mixture was stirred at room temperature for 3 d, then washed with 0.2 mol/l aqueous HCl, sat. aqueous $NaHCO_3$, and brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash column chromatography (hexane : AcOEt=3 : 2 to 2 : 3 v/v) to afford **7** (4.35 g, 13.0 mmol, 87%) as a white solid. ^{1}H-NMR ($CDCl_3$): δ: 7.36—7.51 (m, 5H), 5.29 (s, 1H), 5.03 (q, J=5.1 Hz, 2H), 4.31 (dd, J=15.6, 8.6 Hz, 1H), 2.85 (m,1H), 2.69 (m, 1H), 2.42 (m, 1H), 1.69 (m, 1H), 1.45 (s, 9H). MS (FAB): *m/z* 335 $(M+H)^+$.

3-Amino-1-benzyloxypiperidine-2,6-dione Trifluoroacetate (8) A mixture of trifluoroacetic acid (TFA) (15 ml) and **7** (1.90 g, 5.69 mmol) was stirred at room temperature for 30 min. The reaction mixture was concentrated to afford **8** (3.90 g, quant.) as a pale yellow oil. This intermediate was used for the next reaction without purification. MS (FAB): *m/z* 235 $(M+H)^+$.

4-Benzyloxyphthalic Acid (10a) To a solution of 4-hydroxyphthalic acid **9a** (5.00 g, 27.5 mmol) in 1.7 mol/l aqueous KOH (80 ml, 138 mmol) was added benzyl chloride (3.80 g, 30.0 mmol). The reaction mixture was stirred at 120 °C for 12 h. After the reaction mixture had cooled, 2.0 mol/l aqueous HCl was added to it until the product precipitated. The precipitate was collected by filtration and washed with toluene twice, affording **10a** (8.23 g, quant.) as a white solid. This was used for the next reaction without purification. ^{1}H-NMR (DMSO-d_6): δ: 7.72 (d, J=9.5 Hz, 1H), 7.38 (m, 7H), 5.20 (s, 2H). MS (FAB): *m/z* 273 $(M+H)^+$.

3-Benzyloxyphthalic Acid (10b) To a solution of 3-hydroxyphthalic anhydride **9b** (2.00 g, 12.2 mmol) in 2 mol/l aqueous KOH (30 ml, 60 mmol) was added benzyl chloride (1.65 g, 13.0 mmol). The reaction mixture was stirred at 120 °C for 10 h. After cooling, the mixture was acidified by addition of 2.0 mol/l aqueous HCl, and extracted 5 times with $CHCl_3$/MeOH. The organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated to afford **10b** (2.54 g, 9.34 mmol, 77%) as a white solid. This was used for the next reaction without purification. ^{1}H-NMR (DMSO-d_6): δ: 7.29—7.48 (m, 8H), 5.18 (s, 2H). MS (FAB): *m/z* 273 $(M+H)^+$.

4-Benzyloxyphthalic Anhydride (11a) **10a** (2.20 g, 8.09 mmol) was heated at 200 °C for 1 h. After cooling, the resulting material was purified by recrystallization (AcOEt/hexane) to afford **11a** (1.24 g, 4.88 mmol, 60%) as pale yellow crystals. ^{1}H-NMR (DMSO-d_6): δ: 7.99 (d, J=8.5 Hz, 1H), 7.66 (s, 1H), 7.55 (d, J=8.5 Hz, 1H), 7.48 (d, J=7.2 Hz, 2H), 7.41 (t, J=7.2 Hz, 2H), 7.35 (t, J=7.2 Hz, 1H), 5.35 (s, 2H). MS (FAB): *m/z* 255 $(M+H)^+$.

3-Benzyloxyphthalic Anhydride (11b) This compound was obtained as yellow crystals (80% yield), in a manner similar to that described for the preparation of **11a** from **10b** as a starting material. ^{1}H-NMR (DMSO-d_6): δ: 7.93 (t, J=7.7 Hz, 1H), 7.68 (d, J=7.7 Hz, 1H), 7.59 (d, J=7.7 Hz, 1H), 7.50 (d, J=7.3 Hz, 2H), 7.42 (t, J=7.3 Hz, 2H), 7.35 (t, J=7.3 Hz 1H), 5.41 (s, 2H). MS (FAB): *m/z* 255 $(M+H)^+$.

5-Benzyloxy-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (12a) To a solution of **11a** (270 mg, 1.06 mmol) in dry CH_2Cl_2 (20 ml) was added **5** (200 mg, 0.960 mmol), EDCI·HCl (767 mg, 4.00 mmol), HOBt·H_2O (540 mg, 4.00 mmol), and TEA (607 mg, 6.00 mmol). The reaction mixture was stirred at room temperature for 3 d, then washed twice with 0.2 mol/l aqueous HCl and once with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by flash column chromatography (hexane : AcOEt=3 : 2 v/v) to afford **12a** (343 mg, 0.942 mmol, 98%) as a white solid. ^{1}H-NMR (DMSO-d_6): δ: 11.08 (s, 1H), 7.79 (d, J=8.1 Hz, 1H), 7.37—7.43 (m, 5H), 7.28 (m, 2H), 5.20 (s, 2H), 4.95 (dd, J=12.6, 5.1 Hz, 1H), 2.70—2.92 (m, 3H), 2.15 (m, 1H). MS (FAB): *m/z* 365 $(M+H)^+$.

4-Benzyloxy-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (12b) This compound was obtained as a white solid (89% yield), in a manner similar to that described for the preparation of **12a** from **11b** and **5**. ^{1}H-NMR (DMSO-d_6): δ: 11.09 (s, 1H), 7.82 (t, J=7.7 Hz, 1H), 7.59 (d, J=7.7 Hz, 1H), 7.51 (d, J=7.3 Hz, 2H), 7.46 (d, J=7.7 Hz, 1H), 7.41 (t, J=7.3 Hz, 2H), 7.34 (t, J=7.3 Hz, 1H), 5.37 (s, 2H), 5.08 (dd, J=12.8, 5.4 Hz, 1H), 2.87 (m, 1H), 2.55 (m, 2H), 2.02 (m, 1H). MS (FAB): *m/z* 365 $(M+H)^+$.

2-(1-Benzyloxy-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (12c) To a solution of *N*-phthaloylglutamic anhydride **13** (620 mg, 239 mmol) in dry CH_2Cl_2 (20 ml) was added *O*-benzylhydroxylamine hydrochloride (495 mg, 3.10 mmol), EDCI·HCl (959 mg, 5.00 mmol), HOBt·H_2O (676 mg, 5.00 mmol), and TEA (967 mg, 9.56 mmol). The reaction mixture was stirred at room temperature for 1 d, then washed twice with 0.1 mol/l aqueous HCl and once with brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash column chromatography (hexane : AcOEt=4 : 3 v/v) to afford **12c** (731 mg, 2.01 mmol, 84%) as a white solid. ^{1}H-NMR ($CDCl_3$): δ: 7.88 (m, 2H), 7.76 (m, 2H), 7.53 (m, 2H), 7.35 (m, 3H), 5.04 (s, 2H), 5.00 (dd, J=15.6, 8.6 Hz, 1H), 2.95 (m, 1H), 2.75 (m, 2H), 2.10 (m, 1H). MS (FAB): *m/z* 365 $(M+H)^+$.

5-Benzyloxy-2-(1-benzyloxy-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (12d) This compound was obtained as a white amorphous solid (74% yield), in a manner similar to that described for the preparation of **12a** from **11a** and **8**. ^{1}H-NMR (DMSO-d_6): δ: 7.87 (d, J=8.1 Hz, 1H), 7.36—7.51 (m, 12H), 5.33 (m, 3H), 4.89 (s, 2H), 2.97 (m, 1H), 2.75 (m, 1H), 2.54 (m, 1H), 2.14 (m, 1H). MS (FAB): *m/z* 471 $(M+H)^+$.

4-Benzyloxy-2-(1-benzyloxy-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (12e) This compound was obtained as a white amorphous solid (30% yield), in a manner similar to that described for the preparation of **12a** from **11b** and **8**. ^{1}H-NMR (DMSO-d_6): δ: 7.31—7.59 (m, 13H), 5.38 (s, 2H), 5.21 (dd, J=11.3, 5.2 Hz, 1H), 4.89 (s, 2H), 2.91 (m, 1H), 2.73 (m, 1H), 2.15 (m, 1H), 2.00 (m, 1H). MS (FAB): *m/z* 471 $(M+H)^+$.

5-Hydroxy-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (2a) (5-OH-Thal) To a solution of **12a** (590 mg, 1.62 mmol) in AcOH (15 ml) and AcOEt (40 ml) was added 10% Pd–C (260 mg), and the mixture was stirred at room temperature under an H_2 atmosphere for 1 h. The reaction mixture was filtered through Celite, and the filtrate was concentrated. The residue was purified by flash column chromatography (hexane : AcOEt=3 : 1 to 0 : 1 v/v) to afford **2a** (260 mg, 0.949 mmol, 59%) as a white powder. mp >300 °C. ^{1}H-NMR (DMSO-d_6): δ: 11.06 (s, 1H), 11.01 (s, 1H), 7.73 (d, J=8.1 Hz, 1H),7.16 (s, 1H), 7.14 (d, J=8.1 Hz, 1H), 5.07 (dd, J=12.6, 5.0 Hz, 1H), 2. 87 (m, 1H), 2.55 (m, 2H), 2.02 (m, 1H). MS (FAB): *m/z* 275 $(M+H)^+$. *Anal.* Calcd for $C_{13}H_{10}N_2O_5$: C, 56.94%, H, 3.68%, N, 10.22%. Found: C, 56.82%, H, 3.78%, N, 10.02%.

4-Hydroxy-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (2b) (4-OH-Thal) To a solution of **12b** (500 mg, 1.37 mmol) in MeOH (10 ml) and DMF (10 ml) was added 10% Pd–C (50 mg), and the mixture was stirred at room temperature under an H_2 atmosphere for 4 h, then filtered through Celite, and concentrated. The residue was purified by flash column chromatography (hexane : AcOEt=2 : 3 v/v) to afford **2b** (320 mg, 1.17 mmol, 85%) as a yellow powder. mp 281—282 °C. ^{1}H-NMR (DMSO-d_6): δ: 11.07 (s, 1H), 11.14 (s, 1H), 7.64 (t, J=7.9 Hz, 1H), 7.31 (d, J=7.9 Hz, 1H), 7.24 (d, J=7.9 Hz, 1H), 5.06 (dd, J=12.6, 5.0 Hz, 1H), 2.87 (m, 1H), 2.52 (m, 2H), 2.01 (m, 1H). MS (FAB): *m/z* 275 $(M+H)^+$. *Anal.* Calcd for $C_{13}H_{10}N_2O_5$: C, 56.94%, H, 3.68%, N, 10.22%. Found: C, 56.65%, H, 3.76%, N, 10.06%.

2-(1-Hydroxy-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (2c) (*N*-OH-Thal) This compound was obtained as a white powder (75% yield), in a manner similar to that described for the preparation of **2b** from **12c**. mp 250—251 °C. ^{1}H-NMR (DMSO-d_6): δ: 10.27 (s, 1H), 7.92 (m, 4H), 5.34 (dd, J=12.8, 5.1 Hz, 1H), 3.02 (m, 1H), 2.79 (m, 1H), 2.56 (m, 1H), 2.07 (m, 1H). MS (FAB): *m/z* 275 $(M+H)^+$. *Anal.* Calcd for $C_{13}H_{10}N_2O_5$: C, 56.94%, H, 3.68%, N, 10.22%. Found: C, 56.96%, H, 3.76%, N, 10.17%.

2-(1-Hydroxy-2,6-dioxopiperidin-3-yl)-5-hydroxyisoindoline-1,3-dione (2d) (5,*N*-di-OH-Thal) This compound was obtained as a white powder (88% yield), in a manner similar to that described for the preparation of **2b** from **12d**. mp 250—251 °C. ^{1}H-NMR (DMSO-d_6): δ: 11.08 (s, 1H), 10.26 (s, 1H), 7.75 (d, J=7.7 Hz, 1H), 7.18 (s, 1H), 7.15 (d, J=6.9 Hz, 1H), 5.26 (dd, J=11.3, 5.2 Hz, 1H), 3.00 (m, 1H), 2.75 (m, 1H), 2.53 (m, 1H), 2.03 (m, 1H). MS (FAB): *m/z* 291 $(M+H)^+$. *Anal.* Calcd for $C_{13}H_{10}N_2O_6$: C, 53.80%, H, 3.47%, N, 9.65%. Found: C, 53.56%, H, 3.56%, N, 9.87%.

2-(1-Hydroxy-2,6-dioxopiperidin-3-yl)-4-hydroxyisoindoline-1,3-dione (2e) (4,*N*-di-OH-Thal) This compound was obtained as a white powder (81% yield), in a manner similar to that described for the preparation of **2b** from **12e**. mp 266—267 °C. ^{1}H-NMR (DMSO-d_6): δ: 11.19 (s, 1H), 10.25 (s, 1H), 7.64 (t, J=7.7 Hz, 1H), 7.30 (d, J=7.7 Hz, 1H), 7.24 (d, J=7.7 Hz, 1H), 5.24 (dd, J=13.1, 5.5 Hz, 1H), 3.00 (m, 1H), 2.75 (m, 1H), 2.54 (m, 1H), 2.01 (m, 1H). MS (FAB): *m/z* 291 $(M+H)^+$. *Anal.* Calcd for $C_{13}H_{10}N_2O_6$: C, 53.80%, H, 3.47%, N, 9.65%. Found: C, 53.69%, H, 3.58%, N, 9.54%.

(2*S*,4*R*)-*tert*-Butyl-2-carbamoyl-4-hydroxypyrrolidine-1-carboxylate (15) To a solution of (2*S*,4*R*)-1-*tert*-butoxycar-bonyl-4-hydroxypyrrolidine-2-carboxylic acid **14** (4.90 g, 21.2 mmol) in dry CH_3CN (60 ml) was added EDCI·HCl (4.89 g, 25.5 mmol) and HOBt·H_2O (3.45 g, 25.5 mmol) at 0 °C. The mixture was stirred at room temperature for 6 h, then cooled to 0 °C, and 28% aqueous ammonia (6 ml) was carefully added. Stirring was continued at 0 °C for 30 min and then at room temperature for 1 h. Insoluble material was removed by filtration, then the filtrate was concentrated and purified by flash column chromatography ($CHCl_3$: MeOH=20 : 1 to 5 : 1 v/v) to afford **15** (4.87 g, 21.2 mmol, 100%) as a white solid. ^{1}H-NMR ($CDCl_3$): δ: 6.82, 5.95, 5.35 (each br, 2H), 4.51 (m, 1H), 4.42 (br, 1H), 3.53 (m, 2H), 2.40 (br, 1H), 2.11 (br, 1H), 1.47 (s, 9H). MS (FAB): *m*/*z* 231 $(M+H)^+$. $[\alpha]_D^{22}$ −47.5° (*c*=1.00, MeOH).

(2*S*,4*R*)-*tert*-Butyl-2-carbamoyl-4-*tert*-butyldimethylsilyloxy-pyrrolidine-1-carboxylate (16) To a solution of **15** (100 mg, 0.435 mmol) in dry THF (10 ml) was added *tert*-butyldimethylsilyl chloride (TBDMS-Cl) (73 mg, 0.48 mmol) and imidazole (96 mg, 1.40 mmol) at 0 °C. The mixture was stirred at room temperature for 1 d. Insoluble material was removed by filtration, and the filtrate was concentrated and purified by flash column chromatography (hexane : AcOEt=1 : 3 to 0 : 1 v/v) to afford **16** (130 mg, 0.378 mmol, 87%) as a white solid. ^{1}H-NMR ($CDCl_3$): δ: 6.83, 5.97, 5.27 (each br, 2H), 4.35 (m, 2H), 3.50 (m, 2H), 2.30 (br, 1H), 2.05 (br, 1H), 1.47 (s, 9H), 0.87 (s, 9H), 0.07 (s, 3H), 0.06 (s, 3H). MS (FAB): *m*/*z* 345 $(M+H)^+$. $[\alpha]_D^{22}$ −38.5° (*c*=1.00, MeOH).

(2*S*,4*R*)-*tert*-Butyl-2-carbamoyl-4-*tert*-butyldimethylsilyloxy-5-oxopyrrolidine-1-carboxylate (17) Sodium periodate (6.84 g, 32.0 mmol) was stirred in H_2O (150 ml), and ruthenium(IV) oxide hydrate (1.33 g, 1.00 mmol) was added under an argon atmosphere. The mixture was stirred at room temperature for 5 min, and then a solution of **16** (3.60 g, 10.5 mmol) in AcOEt (90 ml) was added, and the reaction mixture was further stirred at room temperature for 3 d. The aqueous layer was separated and extracted with AcOEt. The combined organic layer was washed with saturated $NaHSO_3$ and brine, dried over $MgSO_4$, and filtered through Celite, and the filtrate was concentrated to afford **17** (3.19 g, 8.91 mmol, 84%) as a white solid. ^{1}H-NMR ($CDCl_3$): δ: 6.01, 5.37 (each br, 2H), 4.62 (dd, *J*=8.1, 2.4 Hz, 1H), 4.49 (d, *J*=9.2 Hz, 1H), 2.54 (dd, *J*=12.8 Hz, 8.1 Hz, 1H), 2.11 (ddd, *J*=12.8, 9.2, 2.4 Hz, 1H), 1.53 (s, 9H), 0.90 (s, 9H), 0.18 (s, 3H), 0.13 (s, 3H). MS (FAB): *m*/*z* 359 $(M+H)^+$. $[\alpha]_D^{22}$ +50.0° (*c*=1.00, MeOH).

(1*S*,3*R*)-*tert*-Butyl-3-*tert*-butyldimethylsilyloxypropyl-carbamate-3-carboxylic Acid (18) A solution of **17** (2.00 g, 5.59 mmol) in dry THF (400 ml) was stirred at 0 °C, and 1.0 mol/l aqueous LiOH (17 ml, 17.0 mmol) was carefully added. The reaction mixture was stirred at 0 °C for 3 h, then acidified by addition of 2.0 mol/l aqueous HCl, and extracted 3 times with $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated to afford **18** (2.30 g, quant.) as a white amorphous solid. This intermediate was used for the next reaction without purification. ^{1}H-NMR (DMSO-d_6): δ: 12.53 (br, 1H), 6.99 (m, 3H), 4.20 (m, 1H), 3.95 (m, 1H), 2.03 (m, 1H), 1.70 (m, 1H), 1.37 (s, 9H), 0.86 (s, 9H), 0.04 (s, 6H). MS (FAB): *m*/*z* 377 $(M+H)^+$. $[\alpha]_D^{22}$ +5.68° (*c*=1.00, MeOH).

(3*S*,5*R*)-*tert*-Butyl-5-*tert*-butyldimethylsilyloxy-2,6-dioxopiperidin-3-ylcarbamate (19) To a solution of **18** (2.30 g, 5.59 mmol) in dry CH_2Cl_2 (300 ml) was added CDI (1.05 g, 6.50 mmol), DMAP (73 mg, 0.600 mmol), and diisopropylethylamine (DIPEA) (1.55 g, 12.0 mmol) at 0 °C. The mixture was stirred at room temperature for 5 d, then washed with 0.5 mol/l aqueous HCl and brine, dried over $MgSO_4$, filtered and concentrated. The residue was purified by flash column chromatography (hexane : AcOEt=3 : 2 v/v) to afford **19** (660 mg, 1.84 mmol, 33%, 2 steps) as a white amorphous solid. ^{1}H-NMR ($CDCl_3$): δ: 7.83 (s, 1H), 5.27 (br, 1H), 4.41 (m, 2H), 2.65 (m, 1H), 2.04 (m, 1H), 1.47 (s, 9H), 0.92 (s, 9H), 0.20 (s, 3H), 0.14 (s, 3H). MS (FAB): *m*/*z* 359 $(M+H)^+$. $[\alpha]_D^{22}$ +18.4° (*c*=1.10, MeOH).

(3*S*,5*R*)-3-Amino-5-*tert*-butyldimethylsilyloxypiperidine-2,6-dione Trifluoroacetate (20) To a stirred solution of **19** (390 mg, 1.09 mmol) in dry CH_2Cl_2 (5 ml) was added TFA (5 ml). The reaction mixture was stirred at room temperature for 30 min, then concentrated to afford **20** (490 mg, quant.) as a pale pink solid. ^{1}H-NMR (DMSO-d_6): δ: 11.42 (s, 1H), 8.42 (br, 3H), 4.65 (dd, *J*=12.2, 4.9 Hz, 1H), 4.34 (dd, *J*=12.8, 4.9 Hz, 1H), 2.36 (m, 1H), 2.06 (ddd, *J*=12.8, 12.8, 12.2 Hz, 1H), 0.88 (s, 9H), 0.12 (s, 3H),0.10 (s, 3H). MS (FAB): *m*/*z* 259 $(M+H)^+$.

2-((3*S*,5*R*)-5-*tert*-Butyldimethylsilyloxy-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (21) (*cis*-5′-OTBDMS-thalidomide) To a solution of **20** (300 mg, 0.806 mmol) in dry CH_2Cl_2 (15 ml) was added phthalic anhydride (148 mg, 1.00 mmol), EDCI·HCl (309 mg, 1.61 mmol), HOBt·H_2O (218 mg, 1.61 mmol), and DIPEA (416 mg, 3.22 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min, then washed with 0.5 mol/l aqueous HCl and brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash column chromatography ($CHCl_3$: MeOH=50 : 1 v/v) to afford **21** (170 mg, 0.438 mmol, 54%) as a white solid. ^{1}H-NMR ($CDCl_3$): δ: 7.95 (s, 1H), 7.93—7.76 (m, 4H), 5.08 (dd, *J*=13.7, 5.1 Hz, 1H), 4.49 (dd, *J*=12.8, 5.6 Hz, 1H), 2.98 (ddd, *J*=13.7, 12.8, 12.8 Hz, 1H), 2.34 (ddd, *J*=12.8, 5.6, 5.1 Hz, 1H), 0.92 (s, 9H), 0.23 (s, 3H), 0.15 (s, 3H). MS (FAB): *m*/*z* 389 $(M+H)^+$. $[\alpha]_D^{22}$ +14.6° (*c*=1.00, $CHCl_3$).

2-(5-*tert*-Butyldimethylsilyloxy-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (24) (*trans*-5′-OTBDMS-Thalidomide) To a solution of **20** (570 mg, 1.53 mmol) in dry CH_2Cl_2 (50 ml) was added phthalic anhydride (252 mg, 1.70 mmol), EDCI·HCl (863 mg, 4.50 mmol), HOBt·H_2O (610 mg, 4.50 mmol), and TEA (608 mg, 6.00 mmol). The reaction mixture was stirred at room temperature for 2 d, then washed with 0.5 mol/l aqueous HCl and brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash column chromatography ($CHCl_3$: MeOH=50 : 1 v/v), followed by recrystallization (CH_2Cl_2/hexane) to afford **24** (151 mg, 0.389 mmol, 25%) as a white solid (single diastereomer of the *trans* form). ^{1}H-NMR ($CDCl_3$): δ: 7.90—7.75 (m, 4H), 7.81 (s, 1H), 5.48 (dd, *J*=12.4, 5.1 Hz, 1H), 4.50 (dd, *J*=1.7, 1.7 Hz, 1H), 2.88 (ddd, *J*=13.5, 12.4, 1.7 Hz, 1H), 2.18 (ddd, *J*=13.5, 5.1, 1.7 Hz, 1H), 0.95 (s, 9H), 0.21 (s, 3H), 0.17 (s, 3H). MS (FAB): *m*/*z* 389 $(M+H)^+$. $[\alpha]_D^{22}$ +34.4° (*c*=1.00, $CHCl_3$).

2-((3*S*,5*R*)-5-Hydroxy-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (*cis*-2f) (*cis*-5′-OH-Thal) To a solution of **21** (90.0 mg, 0.232 mmol) in dry THF (15 ml) was added tetrabutylammonium fluoride (TBAF) (73.0 mg, 0.280 mmol) at 0 °C, and the reaction mixture was stirred at 0 °C. After 90 min, $CHCl_3$ was added, and the whole was washed with 0.5 mol/l HCl and brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by preparative thin layer chromatography ($CHCl_3$: MeOH=10 : 1 v/v) to afford *cis*-**2f** (18.0 mg, 0.0657 mmol, 28%) as a white powder. mp 229—231 °C. ^{1}H-NMR (DMSO-d_6): δ: 11.22 (s, 1H), 7.96—7.88 (m, 4H), 5.82 (d, *J*=6.0 Hz, 1H), 5.29 (dd, *J*=12.8, 5.1 Hz, 1H), 4.54 (ddd, *J*=11.6, 6.0, 5.5 Hz, 1H), 2.49 (m, 1H), 2.28 (ddd, *J*=11.0, 5.5, 5.1 Hz, 1H): MS *m*/*z* 275 $(M+H)^+$. $[\alpha]_D^{22}$ −11.0° (*c*=0.34, MeOH).

2-(5-Hydroxy-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (*trans*-2f) (*trans*-5′-OH-Thal) To a solution of **24** (90.0 mg, 0.232 mmol) in dry THF (15 ml) was added TBAF (73.0 mg, 0.280 mmol) at 0 °C, and the reaction mixture was stirred at 0 °C. After 30 min, $CHCl_3$ was added, and the whole was washed with 0.5 mol/l HCl and brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by recrystallization ($CHCl_3$/MeOH) to afford *trans*-**2f** (38.0 mg, 0.139 mmol, 60%) as a white powder. mp 226—228 °C. ^{1}H-NMR (DMSO-d_6): δ: 11.27 (s, 1H), 7.94—7.88 (m, 4H), 6.52 (d, *J*=4.9 Hz, 1H), 5.15 (dd, *J*=12.0, 5.2 Hz, 1H), 4.30 (ddd, *J*=4.9, 3.5, 3.1 Hz, 1H), 2.65 (ddd, *J*=13.9, 5.2, 3.1 Hz, 1H), 2.17 (ddd, *J*=13.9, 12.0, 3.5 Hz, 1H). MS *m*/*z* 275 $(M+H)^+$. $[\alpha]_D^{22}$ +59.0° (*c*=0.36, MeOH).

5-Benzyloxy-2-(5-*tert*-butyldimethylsilyloxy-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (22) To a solution of **20** (400 mg, 1.08 mmol) in dry CH_2Cl_2 (70 ml) was added **11a** (280 mg, 1.10 mmol), EDCI·HCl (575 mg, 3.00 mmol), HOBt·H_2O (406 mg, 3.00 mmol), and TEA (506 mg, 5.00 mmol). The reaction mixture was stirred at room temperature for 1 d, then washed with 0.5 mol/l aqueous HCl and brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by flash column chromatography (Hexane : AcOEt=2 : 1 v/v) to afford **22** (240 mg, 0.486 mmol, 44%) as a white solid. ^{1}H-NMR ($CDCl_3$): δ: 7.90 (s, 1H), 7.78 (d, *J*=8.1 Hz, 1H), 7.43—7.35 (m, 7H), 5.19 (s, 2H), 5.04 (dd, *J*=12.8, 4.3 Hz, 1H), 4.48 (m, 1H), 2.90 (m, 1H), 2.25 (m, 1H), 0.91 (s, 9H), 0.23 (s, 3H), 0.15 (s, 3H). MS (FAB): *m*/*z* 495 $(M+H)^+$.

5-Benzyloxy-2-(5-hydroxy-2,6-dioxopiperidin-3-yl)-isoindoline-1,3-dione (23) To a solution of **22** (210 mg, 0.425 mmol) in dry THF (20 ml) was added TBAF (122 mg, 0.468 mmol) at 0 °C, and the reaction mixture was stirred at 0 °C for 40 min, and then at room temperature for 2 h. Then $CHCl_3$ was added, and the mixture was washed with 0.5 mol/l HCl and brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by flash column chromatography ($CHCl_3$: MeOH=25 : 1 to 10 : 1 v/v) to afford **23** (166 mg, quant.) as a white solid. ^{1}H-NMR (DMSO-d_6): δ: 11.22 (s, 1H), 7.85 (m, 1H), 7.55—7.33 (m, 7H), 5.80 (d, *J*=4.3 Hz, 1H), 5.33 (s, 2H), 5.24 (m, 1H), 4.52 (m, 1H), 2.49 (m, 1H), 2.25 (m, 1H). MS *m*/*z* 381 $(M+H)^+$.

5-Hydroxy-2-(5-hydroxy-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (2g) (5,5′-di-OH-Thal) To a solution of **23** (160 mg, 0.421 mmol) in dry 1,4-dioxane (20 ml) was added 10% Pd–C (120 mg), and the mixture was stirred at room temperature under an H_2 atmosphere for 5 h, then filtered through Celite, and the filtrate was concentrated *in vacuo*. The residue was purified by flash column chromatography ($CHCl_3$: MeOH=10 : 1 v/v) to af-

ford **2g** (120 mg, 0.144 mmol, 98%) as a white powder. mp 258—260 °C. ^{1}H-NMR (DMSO-d_6): δ: 11.18 (s, 1H), 7.71 (m, 1H), 5.80 (s, 1H), 5.21 (m, 1H), 4.52 (m, 1H), 2.49 (m, 1H), 2.23 (m, 1H). MS (FAB): *m/z* 291 $(M+H)^+$.

Cells and Measurement of TNF-α THP-1 cells were maintained as previously described.[28,29] The exponentially growing cells in RPMI1640 medium supplemented with 10% v/v fetal bovine serum (1.0×10^6 cells/ml) were treated or not treated with 10 nM TPA at 37 °C under a 5% CO_2 atmosphere for 18 h in 24-well multiplates. To test the effects of compounds, cells were treated with TPA (10 nM) in the presence or absence of a test sample at 30 μM. Then the cells were collected by centrifugation (1500 rpm×10 min). The amount of TNF-α in the supernatant was measured by the use of TNF-α Human Biotrak Easy ELISA (Amersham Biosciences, RPN5967) according to the supplier's protocol. The concentration of TNF-α produced by THP-1 cells treated with 10 nM TPA alone was approximately 100 pg/ml under these experimental conditions. The assay was performed at least three times. The results were basically reproducible and a typical set of data (mean values of duplicate) obtained at the same time is presented in Table 1.

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