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

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Putative hydrolyzed metabolites of thalidomide were 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. α-(2-Carboxybenzamido)glutarimide was a more potent TNF-α production inhibitor than thalidomide.

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

Thalidomide (**1**) was developed in the 1950s as a nontoxic sedative/hypnotic drug, but was withdrawn from the market in the early 1960s because of its teratogenicity.[1—5] However, it was subsequently identified as an effective agent for the treatment of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 first for the treatment of erythema nodosum in Hansen's disease in 1998, and then, 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] Although the precise molecular mechanisms of its actions are unknown, thalidomide (**1**) was introduced with data linking it to inhibition of the production of TNF-α. We have already reported the TNF-α production-inhibitory activity of mono- and/or dihydroxylated metabolites of thalidomide.[6] As a part of our continuing work in this area, we also investigated the TNF-α production-inhibitory activity of putative hydrolyzed metabolites of thalidomide.

Thalidomide (**1**) is metabolically labile and many metabolites have been identified or proposed. Both enzymatic hydroxylation and spontaneous hydrolysis occur, with the latter being predominant. Thalidomide's half-life is about 5 h at the physiological pH of 7.4, and twelve hydrolyzed metabolites have been proposed to be formed through successive hydrolysis of amide bond(s)[7,8] (Fig. 1). Although thalidomide has two imide moieties, phthalimide and glutarimide, the phthalimide moiety is more hydrolytically unstable than the glutarimide moiety.[7,8]

In this paper, we describe the synthesis of various candidate metabolites of thalidomide (**2**, **3**, **4**, **6**, **7**, **8**, **10**), as well as the results of chemical and physical characterization and evaluation of their TNF-α production-inhibitory activities.

Chemistry The thalidomide metabolite **2**, formed by hydrolysis at the phthaloyl moiety, was synthesized as shown in Chart 1. Boc-glutamine **14** was treated with 1,1′-carbonyldiimidazole (CDI) in the presence of a catalytic amount of 4-dimethylaminopyridine (DMAP) to afford the cyclic imide **15**.[9] The Boc group of **15** was removed with 30% HBr/AcOH to afford **16** as the HBr salt. The phthalic acid monobenzyl ester **17** was treated with **16** in the presence of methyl chloroformate to afford **18**. The benzyl group of **18** was removed by catalytic hydrogenation with H_2 gas on 10% Pd–C to afford the hydrolyzed thalidomide metabolite **2**.

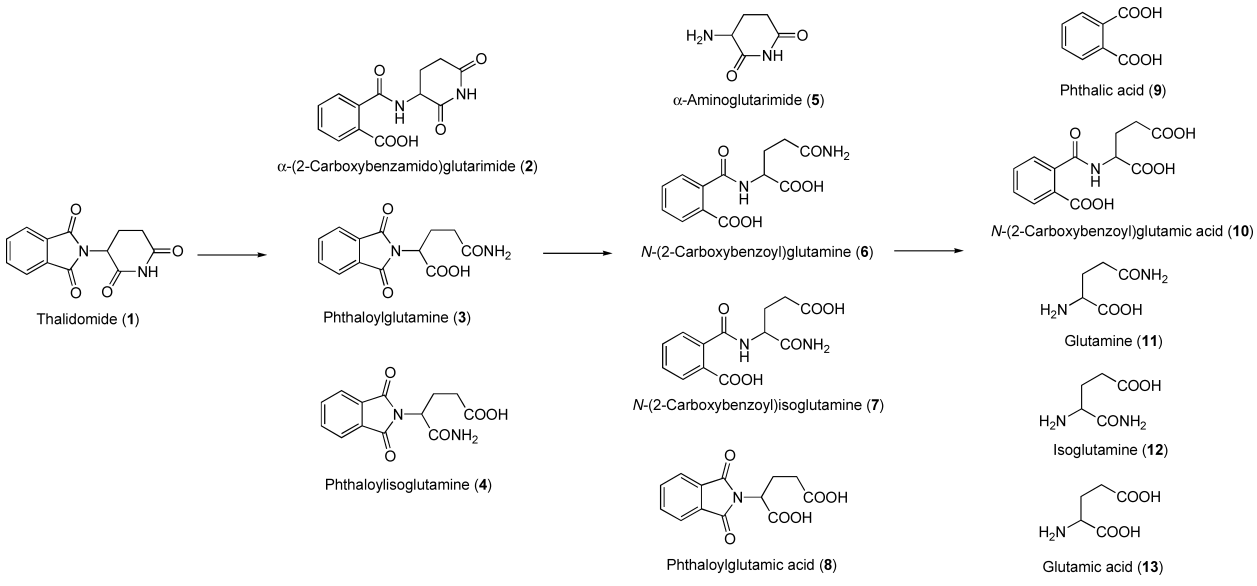

Fig. 1. Hydrolyzed Metabolites of Thalidomide

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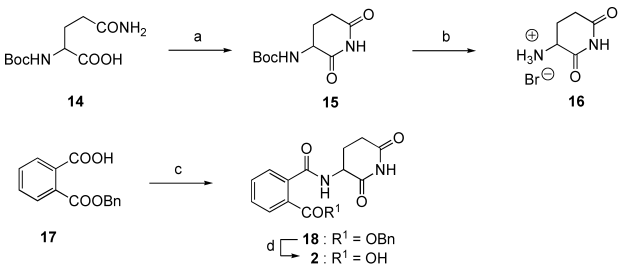
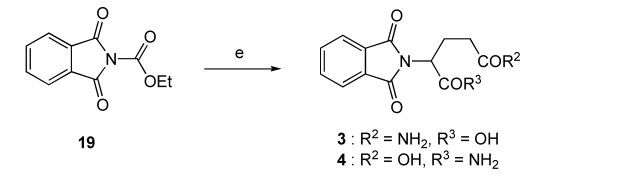


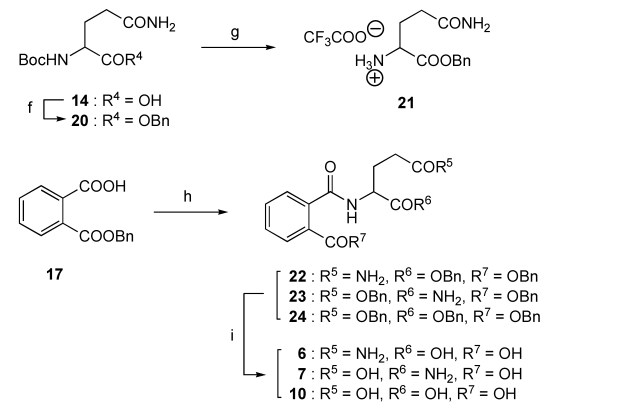
Reagents and conditions: (a) CDI, DMAP, TEA, THF, rt, 3 days (90%) ; (b) 30% HBr /AcOH, rt, 30 min (98%) ; (c) i) methyl chloroformate, TEA, THF, 0ºC, 5 min, ii) **16**, 0ºC to rt, 18 h (50%) ; (d) H_2, 10% Pd-C, 1,4-dioxane, rt, 2 h (80%).

Chart 1



Reagents and conditions: (e) glutamine or isoglutamine, Na_2CO_3, H_2O, 0ºC to rt, 1 h (18–49%)

Chart 2



Reagents and conditions: (f) BnBr, TEA, THF, 0ºC to rt, 20 h (61%) ; (g) TFA, CH_2Cl_2, rt, 90 min (quant.) ; (h) i) methyl chloroformate, TEA, THF, 0ºC, 30 min, ii) **21** or H-Glu(OBn)-NH_2·HCl or H-Glu(OBn)-OBn·Tos-OH, 0ºC to rt, 3 h (5–30%) ; (i) H_2, 10% Pd-C, 1,4-dioxane, rt, 3–8 h (99%–quant.).

Chart 3

Thalidomide metabolites **3** and **4**, generated by hydrolysis at the glutarimide moiety, were synthesized as shown in Chart 2, by treating *N*-ethoxycarbonylphthalimide **19** with glutamine or isoglutamine, respectively, in the presence of sodium carbonate.[10] Phthaloylglutamic acid **8** was purchased from Tokyo Kasei Kogyo Co., Ltd., Japan.

Thalidomide metabolites **6**, **7**, and **10**, hydrolyzed at both the glutarimide moiety and the phthaloyl moiety, were synthesized as shown in Chart 3. Protection of the carboxyl group of the Boc-glutamine **14** as the benzyl ester **20**, followed by removal of the Boc group, afforded the glutamine benzyl ester **21** as the trifluoroacetic acid (TFA) salt.[11] Phthalic acid monobenzyl ester **17** was treated with **21**, isoglutamine benzyl ester, or glutamic acid dibenzyl ester in the presence of methyl chloroformate to afford **22**, **23**, and **24**. The benzyl groups of **22**, **23**, and **24** were removed by catalytic hydrogenation with H_2 gas on 10% Pd–C to afford metabolites **6**, **7**, and **10**.

Table 1. TNF-α Production-Inhibitory Activity of Thalidomide (**1**) and Its Hydrolyzed Metabolites at 3 μM

Compound	TNF-α production-inhibitory activity (%)
Thalidomide (**1**)	32
α-(2-Carboxybenzamido)glutarimide (**2**)	80
Phthaloylglutamine (**3**)	13
Phthaloylisoglutamine (**4**)	18
α-Aminoglutarimide (**5**)	13
N-(2-Carboxybenzoyl)glutamine (**6**)	41
N-(2-Carboxybenzoyl)isoglutamine (**7**)	44
Phthaloylglutamic acid (**8**)	48
Phthalic acid (**9**)	9
N-(2-Carboxybenzoyl)glutamic acid (**10**)	62
Glutamine (**11**)	*ca.* 0
Isoglutamie (**12**)	*ca.* 0
Glutamic acid (**13**)	1

TNF-α Production-Inhibitory Activity TNF-α is one of the cytokines mediating immune regulation, and has a wide range of activities.[12,13] The growing understanding of the pathophysiological role of TNF-α in various diseases, as well as the discovery of the TNF-α production-inhibitory activity of thalidomide (**1**),[14] has led researchers to regard this activity as the molecular basis for the pharmacological effects elicited by thalidomide (**1**).[15] 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 metabolically labile, 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 putative hydrolyzed metabolites of thalidomide (**2**—**4**, **6**—**8**, **10**) by means of a previously reported method using a human monocytic leukemia cell line, THP-1.[16,17] THP-1 cells do not produce TNF-α under normal cell culture conditions, but do produce TNF-α in response to stimulation with 12-*O*-tetradecanoylphorbol 13-acetate (TPA).[16,17] Thalidomide (**1**) shows moderate TNF-α production-inhibitory activity in this assay system.[1—5,16,17] The TNF-α production-inhibitory activity of test compounds was measured as described in the experimental section as %-inhibition values, where 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 with test compounds at the concentration of 3 μM is presented in Table 1.

The mother compound, thalidomide (**1**), showed moderate TNF-α production-inhibitory activity (32% inhibition at 3 μM), in accordance with our previous reports.[16,17] Ring opening by hydrolysis at the phthalimide moiety (**2**) caused powerful enhancement of the activity (80% inhibition). On the other hand, ring opening at the glutarimide moiety (**3**, **4**) decreased the activity (13—18%). Ring opening at both the phthalimide and the glutarimide moiety (**6**, **7**) caused slight enhancement of the activity (41—44%). Though two kinds of amino acid derivatives, glutamine and isoglutamine deriv-

atives, are produced by hydrolysis at the glutarimide moiety, the isoglutamine derivatives (**4**, **7**) showed slightly higher activity than the glutamine derivatives (**3**, **6**). Interestingly, although glutarimide ring opening (**3**, **4**) decreased the activity, further hydrolysis at distal primary amide (**8**) resulted in recovery of the activity (48% inhibition). Similarly, the activities of the phthalimide and glutarimide ring-opened metabolites (**6**, **7**) were enhanced by further hydrolysis at the distal primary amide (**10**) (62%). Final hydrolysate, *i.e.*, α-amino acid derivatives (**11**—**13**) and phthalic acid (**9**) showed no or slight activity (0—9%).

The results indicate that spontaneous hydrolysis of thalidomide (**1**) may have various effects (loss, remaining, decrease and increase) on TNF-α production-inhibitory activity. It appears that the metabolism of thalidomide (**1**) might not have a dramatic effect on its TNF-α production-inhibitory activity, because some metabolites retain the effects and increase of the effect found in some metabolites is not drastic. Investigation of the effects of metabolism on other biological activities is under way.

Experimental

***tert*-Butyl 2,6-Dioxopiperidin-3-ylcarbamate (15)** To a solution of Boc-Gln-OH **14** (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 **15** (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 (16) A mixture of **15** (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 **16** (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 Hz, 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 $(M+H)^+$.

α-(2-Benzyloxycarbonylbenzamido)glutarimide (18) To a solution of phthalic acid monobenzyl ester **17** (300 mg, 1.17 mmol) in dry THF (50 ml) was added methyl chloroformate (144 mg, 1.50 mmol) and TEA (485 mg, 4.80 mmol) at 0 °C. The mixture was stirred at 0 °C for 15 min, and **16** (500 mg, 2.40 mmol) was added. The reaction mixture was stirred at 0 °C for 45 min and room temperature for 4 h, then AcOEt was added, and the mixture was washed with 0.2 mol/l aqueous HCl, brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash column chromatography (hexane : AcOEt=2 : 3 to $CHCl_3$: acetone=1 : 1 v/v) to afford **18** (160 mg, 0.437 mmol, 37%) as a white solid. ^{1}H-NMR ($CDCl_3$): δ 7.99 (d, *J*=7.7 Hz, 1H), 7.82 (s, 1H), 7.58 (t, *J*=7.6 Hz, 1H), 7.52 (d, *J*=7.6 Hz, 1H), 7.50 (t, *J*=7.6 Hz, 1H), 7.33—7.42 (m, 5H), 6.54 (d, *J*=4.7 Hz, 1H), 5.30 (d, *J*=5.3 Hz, 2H), 4.31 (ddd, *J*=12.8, 4.7, 4.7 Hz, 1H), 2.61—2.80 (m, 3H), 1.72 (m, 1H). MS (FAB): *m*/*z* 367 $(M+H)^+$.

α-(2-Carboxybenzamido)glutarimide (2) To a solution of **18** (300 mg, 0.820 mmol) in dry 1,4-dioxane (15 ml) was added 10% Pd–C (60 mg). The mixture was stirred at room temperature under an H_2 atmosphere for 2 h, then filtered through Celite, and the filtrate was concentrated. The residue was purified by flash column chromatography ($CHCl_3$: MeOH=20 : 1 to 6 : 1 v/v) to afford **2** (180 mg, 0.652 mmol, 80%) as a white powder. mp 86—88 °C. ^{1}H-NMR (DMSO-d_6): δ 12.93 (s, 1H), 10.81 (s, 1H), 8.61 (d, *J*=8.4 Hz, 1H), 7.77 (d, *J*=7.8 Hz, 1H), 7.60 (t, *J*=7.8 Hz, 1H), 7.51 (t, *J*=7.8 Hz, 1H), 7.46 (d, *J*=7.8 Hz, 1H), 4.69 (ddd, *J*=17.1, 8.4, 8.4 Hz, 1H), 2.75 (m, 1H), 2.53 (m, 1H), 2.00 (m, 2H). MS (FAB): *m*/*z* 276 $(M+H)^+$. *Anal.* Calcd for $C_{13}H_{12}N_2O_5$: C, 56.52%, H, 4.38%, N, 10.14%. Found: C, 56.50%, H, 4.53%, N, 10.03%.

Phthaloylglutamine (3) To a solution of glutamine (2.00 g, 13.7 mmol) in 0.55 mol/l aqueous Na_2CO_3 (30 ml, 16.4 mmol) was added *N*-ethoxycarbonylphthalimide **19** (4.40 g, 20.0 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 10 min and at room temperature for 50 min, then insoluble material was removed by filtration and 2.0 mol/l aqueous HCl was added to the filtrate until the pH reached about 2. Then the mixture was stirred at 0 °C for 30 min, the solution was removed and the remaining sticky solid was purified by flash column chromatography (AcOEt : AcOH=30 : 1 v/v) to afford **3** (1.85 g, 6.70 mmol, 49%) as a white solid. mp 159—160 °C. ^{1}H-NMR (DMSO-d_6): δ 13.14 (s, 1H), 7.86—7.92 (m, 4H), 7.16 (s, 1H), 6.88 (s, 1H), 4.74 (dd, *J*=10.7, 4.3 Hz, 1H), 2.35 (m, 1H), 2.25 (m, 1H), 2.08 (m, 2H). MS (FAB): *m*/*z* 277 $(M+H)^+$. *Anal.* Calcd for $C_{13}H_{12}N_2O_5$: C, 56.52%, H, 4.38%, N, 10.14%. Found: C, 56.37%, H, 4.39%, N, 10.07%.

Phthaloylisoglutamine (4) This compound was obtained as a white solid (18% yield) in a manner similar to that described for the preparation of **3**, but from isoglutamine as a starting material. mp 119—121 °C. ^{1}H-NMR (DMSO-d_6): δ 12.04 (s, 1H), 7.83—7.88 (m, 4H), 7.57 (s, 1H), 7.16 (s, 1H), 4.59 (dd, *J*=10.3, 4.3 Hz, 1H), 2.37 (m, 1H), 2.20 (m, 3H). MS (FAB): *m*/*z* 277 $(M+H)^+$. *Anal.* Calcd for $C_{13}H_{12}N_2O_5$: C, 56.52%, H, 4.38%, N, 10.14%. Found: C, 56.24%, H, 4.40%, N, 10.01%.

***N*-*tert*-Butoxycarbonylglutamine Benzyl Ester (20)** To a solution of Boc-Gln-OH **14** (2.00 g, 8.12 mmol) in dry THF (50 ml) was added benzyl bromide (1.53 g, 8.94 mmol) and TEA (911 mg, 9.00 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 2 h and at room temperature for 18 h, then $CHCl_3$ was added, and whole was washed with 0.2 mol/l aqueous HCl, sat. $NaHCO_3$ and brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash column chromatography (hexane : AcOEt=1 : 2 to 0 : 1 v/v) to afford **20** (1.65 g, 4.91 mmol, 61%) as a white solid. ^{1}H-NMR ($CDCl_3$): δ 7.33—7.37 (m, 5H), 6.01 (s, 1H), 5.31 (m, 2H), 5.18 (m, 2H), 4.36 (m, 1H), 2.19—2.34 (m, 3H), 1.94 (m, 1H), 1.44 (s, 9H). MS (FAB): *m*/*z* 337 $(M+H)^+$.

Glutamine Benzyl Ester Trifluoroacetate (21) A mixture of **20** (400 mg, 1.19 mmol), CH_2Cl_2 (6 ml) and TFA (6 ml) was stirred at room temperature for 90 min. The reaction mixture was concentrated to afford **21** (550 mg, quant.) as a pale yellow oil. This intermediate was used for the next reaction without purification. MS (FAB): *m*/*z* 237 $(M+H)^+$.

***N*-(2-Benzyloxycarbonylbenzoyl)glutamine Benzyl Ester (22)** This compound was obtained as a white amorphous solid (19% yield) in a manner similar to that described for the preparation of **18**, but from **17** and **21** as starting materials. The mobile phase solvent for flash column chromatography was AcOEt : $CHCl_3$: MeOH=1 : 1 : 0 to 25 : 25 : 1 v/v/v. ^{1}H-NMR ($CDCl_3$): δ 7.95 (d, *J*=7.3 Hz, 1H), 7.56 (t, *J*=7.3 Hz, 1H), 7.48 (m, 2H), 7.33—7.41 (m, 10H), 6.61 (d, *J*=8.1 Hz, 1H), 6.37 (s, 1H), 5.30 (s, 2H), 5.26 (s, 1H), 5.20 (m, 2H), 4.81 (m, 1H), 2.47 (m, 1H), 2.39 (m, 1H), 2.27 (m, 1H), 1.98 (m, 1H). MS (FAB): *m*/*z* 475 $(M+H)^+$.

***N*-(2-Benzyloxycarbonylbenzoyl)isoglutamine Benzyl Ester (23)** This compound was obtained as a white solid (46% yield) in a manner similar to that described for the preparation of **18**, but from **17** and H-Glu(OBn)-NH_2·HCl as starting materials. The mobile phase solvent for flash column chromatography was hexane : $CHCl_3$: MeOH=1 : 1 : 0 to 20 : 20 : 1 v/v/v. ^{1}H-NMR ($CDCl_3$): 7.99 (dd, *J*=7.7, 1.3 Hz, 1H), 7.54 (dt, *J*=7.7, 1.3 Hz, 1H), 7.48 (dt, *J*=7.7, 1.3 Hz, 1H), 7.30—7.42 (m, 11H), 7.19 (s, 1H), 6.81 (d, *J*=8.0 Hz, 1H), 5.41 (s, 1H), 5.30 (m, 2H), 5.09 (m, 2H), 5.20 (m, 2H), 4.66 (ddd, *J*=8.0, 4.3, 4.3 Hz, 1H), 2.70 (m, 1H), 2.54 (m, 1H), 2.33 (m, 1H), 2.13 (m, 1H). MS (FAB): *m*/*z* 475 $(M+H)^+$.

***N*-(2-Benzyloxycarbonylbenzoyl)glutamic Acid Dibenzyl Ester (24)** This compound was obtained as a white solid (5.4% yield) in a manner similar to that described for the preparation of **18**, but from **17** and H-Glu(OBn)-OBn·Tos-OH as starting materials. The mobile phase solvent for flash column chromatography hexane : AcOEt=1 : 1 v/v. ^{1}H-NMR ($CDCl_3$): 7.92 (d, *J*=8.1 Hz, 1H), 7.43—7.53 (m, 3H), 7.28—7.38 (m, 15H), 6.53 (d, *J*=7.3 Hz, 1H), 5.25 (m, 2H), 5.17 (m, 2H), 5.08 (s, 2H), 4.77 (m, 1H), 2.39—2.54 (m, 2H), 2.30 (m, 1H), 2.09 (m, 1H). MS (FAB): *m*/*z* 566 $(M+H)^+$.

***N*-(2-Carboxybenzoyl)glutamine (6)** This compound was obtained as a white amorphous solid (quantitative yield) in a manner similar to that described for the preparation of **2**, but from **22** as a starting material. mp 98—100 °C. ^{1}H-NMR (DMSO-d_6): δ 8.14 (d, *J*=7.7 Hz, 1H), 7.24 (d, *J*=7.7 Hz, 1H), 7.08 (t, *J*=7.7 Hz, 1H), 7.01 (t, *J*=7.7 Hz, 1H), 6.95 (d, *J*=7.7 Hz, 1H), 6.69 (s, 1H), 6.28 (s, 1H), 3.83 (ddd, *J*=7.7, 7.7, 4.7 Hz, 1H), 1.71 (m, 2H), 1.53 (m, 1H), 1.36 (m, 1H). MS (FAB): *m*/*z* 295 $(M+H)^+$.

***N*-(2-Carboxybenzoyl)isoglutamine (7)** This compound was obtained as a white amorphous solid (98% yield) in a manner similar to that described for the preparation of **2**, but from **23** as a starting material. mp 90—93 °C. ^{1}H-NMR (DMSO-d_6): δ 12.85 (br s, 2H), 8.50 (d, *J*=7.9 Hz, 1H), 7.82 (d, *J*=7.9 Hz, 1H), 7.58 (t, *J*=7.9 Hz, 1H), 7.51 (t, *J*=7.7 Hz, 1H), 7.42 (d,

J=7.7 Hz, 1H), 7.35 (s, 1H), 7.20 (s, 1H), 4.28 (m, 1H), 2.32 (m, 2H), 2.08 (m, 1H), 1.75 (m, 1H). MS (FAB): m/z 295 $(M+H)^+$.

***N*-(2-Carboxybenzoyl)glutamic Acid (10)** This compound was obtained as a hygroscopic white amorphous solid (99% yield) in a manner similar to that described for the preparation of **2**, but from **24** as a starting material. ^{1}H-NMR (DMSO-d_6): δ 12.67 (br s, 3H), 8.71 (d, J=7.3 Hz, 1H), 7.86 (d, J=7.3 Hz, 1H), 7.68 (t, J=7.3 Hz, 1H), 7.61 (t, J=7.3 Hz, 1H), 7.53 (d, J=7.3 Hz, 1H), 4.48 (m, 1H), 2.40—2.61 (m, 2H), 1.98—2.19 (m, 2H). MS (FAB): m/z 296 $(M+H)^+$.

Cells and Measurement of TNF-α THP-1 cells were maintained as previously described.[11,12] 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 3 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 the experimental conditions. 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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