Novel Biological Response Modifiers: Phthalimides with Tumor Necrosis Factor-α Production-Regulating Activity

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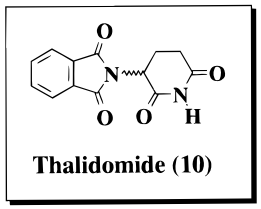
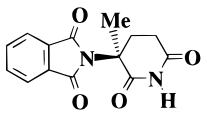
Novel N-substituted phthalimides (2-substituted 1*H*-isoindole-1,3-diones) were prepared, and their effects on tumor necrosis factor-α (TNF-α) production by human leukemia cell line HL-60 stimulated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or okadaic acid (OA) were examined. A structure−activity relationship study of the *N*-phenylphthalimides and *N*-benzylphthalimides revealed that their enhancing effect on TPA-induced TNF-α production by HL-60 cells and their inhibiting effect on OA-induced TNF-α production by HL-60 cells are only partially correlated.

Introduction

Tumor necrosis factor-α (TNF-α), an important cytokine produced by activated monocytes/macrophages, was originally identified as an endotoxin-induced serum factor that causes hemorragic necrosis of transplanted solid tumors.[1] Because TNF-α exhibits striking cytotoxicity selectively against various tumor cells, it has attracted attention as a potential antitumor drug.[2] Investigation of the biological effects elicited by TNF-α has revealed that it is a pleiotropic cytokine with numerous effects on mammalian cells, and its actions are initiated by binding to high-affinity receptors.[3] Though TNF-α plays a critical role in certain physiological immune systems, it causes severe damage to the host when produced in large excess. Therefore, TNF-α can be regarded as possessing both favorable and unfavorable effects. The favorable effects include direct tumor-killing effect,[2] stimulation of the host's immune system,[4] and action as a growth factor for normal B-cells.[5] The unfavorable effects include induction of tissue inflammation,[6] tumor-promoting action,[7] stimulation of human immunodeficiency virus (HIV) replication,[8] and induction of insulin resistance.[9] These pleiotropic effects of TNF-α indicate that TNF-α production enhancers in some cases and production inhibitors in other cases would be useful as biological response modifiers (BRMs) under various circumstances.[10] Moreover, tissue- and/or cell-type-specific regulation of TNF-α production would be useful, since TNF-α is rapidly cleared from the circulation.

A possible lead compound for the above purpose is thalidomide [*N*-α-phthalimidoglutarimide (**10**) (Figure 1)]. Thalidomide was developed as a hypnotic/sedative agent but had to be withdrawn from the market because of its teratogenicity. In spite of this, there has been a resurgance of interest in the drug in recent years due to its potential usefulness for the treatment of acquired immunodeficiency syndrome (AIDS),[8] graft-*versus*-host disease (GVHD),[11] leprosy,[12] and other related diseases.[13] The effectiveness of the drug in these diseases has been attributed to its specific inhibitory activity on TNF-α production.[14] However, we found recently that the regulation by thalidomide of TNF-α production is specific to cell type and to inducer, *i.e.*, (1) thalidomide enhances TPA-induced TNF-α production by human leukemia HL-60 cells, while it inhibits TPA-induced TNF-α production by another human leukemia cell line, THP-1, and (2) it inhibits TNF-α production by both HL-60 and THP-1 cells when the cells are stimulated with okadaic acid (OA).[15]





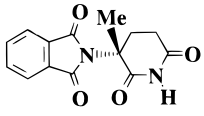


Figure 1. Structures of thalidomide, (*S*)-methylthalidomide, and (*R*)-methylthalidomide.

On the basis of these findings, we have been engaged in structural modification of thalidomide with the aim of creating superior regulators of TNF-α production. For the evaluation of the compounds obtained, TNF-α production-enhancing activity was assayed using HL-60 stimulated with TPA, and TNF-α production-inhibiting activity was assayed using HL-60 stimulated with OA. Studies on structural simplification of the glutarimide moiety of thalidomide afforded potent bidirectional TNF-α production regulators, such as 2-(2,6-diisopropylphenyl)-4,5,6,7-tetrafluoro-1*H*-isoindole-1,3-dione [FPP-33 (**30**)] (Table 5) and 2-(2,6-diisopropylphenyl)-1-oxo-3-thioxo-1*H*-isoindole [PPS-33 (**18**)] (Table 3).[16] Both compounds enhanced TPA-induced TNF-α production in the nanomolar/micromolar concentration range,

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while they completely inhibited OA-induced TNF-α production in the same concentration range.[16]

More recently, we also found a clear enantiodependence of the inducer-specific bidirectional TNF-α production-regulating activity. In the case of optically active thalidomide analogues, (*S*)- and (*R*)-α-methylthalidomide (Figure 1), only the (*S*)-form shows TNF-α production-enhancing activity in the TPA/HL-60 assay system. In contrast, the (*R*)-form shows much more potent TNF-α production-inhibiting activity than the (*S*)-form in the OA/HL-60 assay system.[17] On the basis of this observation, we anticipated that separation of the bidirectional TNF-α production-regulating activities could be achieved by the use of optically active phthalimides. We found that optically active tetrafluorophthalimides, (*R*)-FPTP (**33**) and (*R*)-FPTN (**35**) (Table 6), exhibited potent TNF-α production-inhibiting activity in the nanomolar concentration range without concomitant TNF-α production-enhancing activity.[18]

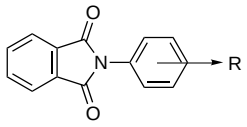
In this paper we would like to describe the structure−activity relationships of novel *N*-phenyl- and *N*-benzylphthalimides, based on assays of TPA-induced TNF-α production-enhancing activity and OA-induced TNF-α production-inhibiting activity.

Results

Assay Systems. The HL-60 cells used produce no detectable amount of TNF-α under the usual culture conditions, but begin to produce TNF-α after treatment with TPA or OA. The induction of TNF-α by TPA and OA was dose-dependent, and we chose TPA and OA concentrations of 10 and 50 nM respectively, because the effects of the test compounds were clearly apparent at these concentrations of TPA/OA. The amount of TNF-α produced by HL-60 cells under our standard experimental conditions (5×10^5 cells/mL, incubated in the presence of 10 nM TPA or 50 nM OA for 16 h) was 150−180 or 1000−1200 pg/mL, respectively, and the value separately determined in each set of experiments was taken as 100%. The effect of a compound was represented as the amount (%) of TNF-α produced by HL-60 cells in the co-presence of TPA or OA and the test compound. The amount of TNF-α produced by TPA/OA-stimulated HL-60 cells in the presence of test compounds showed some variation from experiment to experiment. However, the order of efficacy of the test compounds was reproducible. Therefore, typical data obtained in experiments performed at the same time are presented. In each set of experiments, the assay was performed in duplicate or triplicate (the mean value was taken) and at least three times. A typical set of data obtained at the same time is presented in each table and figure. None of the phthalimides described in this paper induced TNF-α production by themselves in the concentration range investigated. Cell numbers were counted at the time when the amount of TNF-α was measured. Almost no difference in the cell numbers between incubation mixtures in the presence and absence of the test compound was observed in the concentration range investigated.

To assess the cell toxicity of the compounds, human embryonic lung fibroblast WI-38 cells were treated with various concentrations of test compounds at 37 °C for 48 h. After the incubation, the viability of the treated cells was measured by testing the succinate−tetrazolium reductase system using the WST-1 method.[19] The

Table 1. Effects of Monosubstituted *N*-Phenylphthalimides on TNF-α Production



compd	code (30 μM)	R	amount of TNF-α (%)[a] TPA/HL-60	amount of TNF-α (%)[a] OA/HL-60	cytotoxicity[b] IC_{50} (μg/mL)
1	PP-00	H	100	61	>100
2	PP-10	2-Me	100	68	>100
3	PP-n50	2-nPen	151	35	>100
4	PP-30	2-iPr	158	105	>100
5	PP-ph0	2-Ph	262	40	>100
6	PP-M0	2-MeO	95	21	>100
7	PP-N0	2-NO_2	89	53	>100
8	PP-03	3-iPr	158	105	>100
9	PP-003	4-iPr	139	33	>100
10	thalidomide		135	58	>100

[a] HL-60 cells were treated with 10 nM TPA or 50 nM OA in the presence of 30 μM test compound. The amount of TNF-α secreted in the culture medium was measured by ELISA. The amount of TNF-α production in the presence of 10 nM TPA alone or 50 nM OA alone was defined as 100%. [b] WI-38 cells were treated with test compounds, and cell viability was determined by the WST-1 method as described in the text.

IC_{50} values determined are presented in Tables 1−6. As shown in the tables, only a poor correlation was observed between the TNF-α production-regulating activity (especially TNF-α production-inhibiting activity) and the cell toxicity of the compounds. For example, both PP-33 (**17**) and 4APP-33 (**25**) are potent TNF-α production inhibitors on OA-stimulated HL-60 cells (21%, Table 4), but the latter is much more toxic (IC_{50} = 25 μM) than the former (IC_{50} = >100 μg/mL, corresponding to >322 μM) (Table 4). In addition, FPP-33 (**30**), which is highly toxic (IC_{50} = 2.3 μM, Table 5), and PPS-33 (**18**), which is less toxic (IC_{50} = 95 μM, Table 3), both showed a potent TNF-α production-enhancing effect on TPA-stimulated HL-60 cells. These results indicate that the TNF-α production-regulating activity of these compounds is independent of their toxic effect, at least in part.

Effects of *Ortho* Substituents at the *N*-Phenyl Group. First, we investigated the effects of *ortho* substituents introduced at the *N*-phenyl moiety of *N*-phenylphthalimide on TPA- and OA-induced TNF-α production by HL-60 cells (Table 1).[20] Our preliminary experiments revealed that several active compounds showed activity at the concentration range 1−100 μM. Therefore, all of the compounds were assessed at 30 μM concentration. As previously reported, unsubstituted *N*-phenylphthalimide (PP-00: **1**) lacks TNF-α production-enhancing activity, but introduction of alkyl substituents at the *ortho* position of the phenyl moiety causes the appearance of the activity. In a series of *ortho*-substituted compounds, the enhancing activity increased in the order of H (PP-00: **1**, 100%) = Me (PP-10: **2**, 100%) < *n*-Pen (PP-n50: **3**, 151%) < *i*-Pr (PP-30: **4**, 158%) < Ph (PP-ph0: **5**, 262%), indicating that the steric bulk of the substituents plays a critical role in the TNF-α production-enhancing activity. On the other hand, the electronic factor of the substituent had little or no effect: the MeO (PP-M0: **6**) and NO_2 (PP-N0: **7**) substituted compounds did not exhibit TNF-α production-enhancing activity.

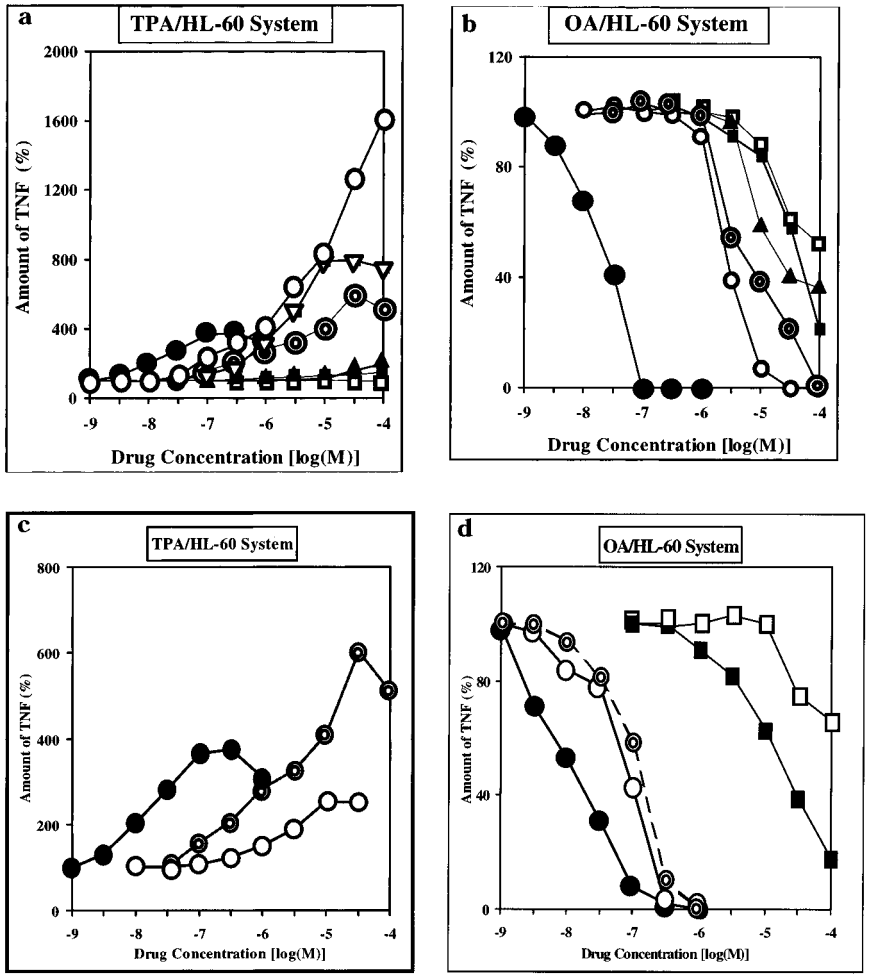


Figure 2. Dose−response curves of typical phthalimide derivatives. Horizontal scale: concentration of added test compound. Vertical scale: amount of TNF-α (%, vide infra). (a and c) TPA-induced TNF-α production-enhancing activity. HL-60 cells were treated with 10 nM TPA in the presence of a test compound. The amount of TNF-α produced by HL-60 cells stimulated with 10 nM TPA alone was defined as 100%. (b and d) OA-induced TNF-α production-inhibiting activity. HL-60 cells were treated with 50 nM OA in the presence of a test compound. The amount of TNF-α produced by HL-60 cells stimulated with 50 nM OA alone was defined as 100%. Part a: (■) thalidomide, (□) PP-00 (**1**), (▲) PP-11 (**11**), (two concentric circles) PP-33 (**17**), (○) PPS-33 (**18**), (▽) 4NPP-33 (**23**), (●) FPP-33 (**30**). Part b: (■) thalidomide, (□) PP-00 (**1**), (▲) PP-11 (**11**), (two concentric circles) PP-33 (**17**), (○) PPS-33 (**18**), (●) FPP-33 (**30**). Part c: (●) FPP-33 (**30**), (two concentric circles) PP-33 (**17**), (○) ClPP-33 (**31**). Part d: (●) (*R*)-FPTP (**33**), (○) (*R*)-FPTN (**35**), (two concentric circles) (*R*)-FPTH (**37**), (■) (*R*)-methylthalidomide, (□) (*S*)-methylthalidomide.

As for TNF-α production-inhibiting activity, PP-00 (**1**) exhibited moderate activity (61%), comparable to that of thalidomide (**10**) (58%) (Table 1). In a series of *ortho*-substituted compounds, we could not find any clear substituent effect on the TNF-α production-inhibiting activity. The dose−response curves for the enhancing and inhibiting activities of typical compounds on TNF-α production are shown in Figure 2a,b.

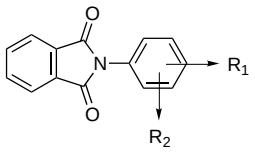
Effects of Dialkyl Substituents at the *N*-Phenyl Group. Because TNF-α production-enhancing activity was suspected to depend on the steric factor of the substituents at the *N*-phenyl moiety, we next investigated *N*-(dialkyl-substituted phenyl)phthalimides at the concentration of 30 μM (Table 2).[20] As previously reported, *o*,*o*′-dimethylation (PP-11: **11**) caused the appearance of the TNF-α production-enhancing activity (161%). The *ortho* positions seem to be the best at which to introduce two methyl groups to obtain potent enhancing activity, *i.e.*, PP-11 (**11**) was more active than the *m*,*m*′-dimethylated analog (PP-0101: **12**, 136%), although the latter compound was moderately active. As was the case among *ortho*-monoalkylated compounds, the steric bulk of the two alkyl groups introduced seems to be critical, and the *o*,*o*′-diisopropyl derivative (PP-33: **17**) exhibited potent TNF-α production-enhancing activity amounting to ca. 600 % at 30 μM in this experiment (Table 2).

Interestingly, the TNF-α production-inhibiting activity showed similar but not identical structure−activity relationships. PP-33 (**17**) showed the strongest inhibitory activity on OA-induced TNF-α production HL-60 cells (21%) among the *o*,*o*′-dialkylated analogues listed in Table 2. Though the inhibitory activity decreased in the order of PP-33 (**17**, 21%) > PP-11 (**11**, 40%) > PP-00 (**1**, 61%) (Table 2: the same order as that of the enhancing activity on TPA-induced TNF-α production by the same cell line), PP-22 (**14**) exceptionally showed inhibitory activity (53%) slightly weaker than that of PP-11 (**11**, 40%). The effective concentration ranges of these compounds are roughly the same for the TPA-induced HL-60 and OA-induced HL-60 assay systems (1−100 μM, Figure 2a,b).

Effects of Variation of the Succinimide Moiety. Next, we investigated the effect of modifying the succinimide moiety.[21] For this purpose, the 2,6-diisopropylphenyl group was used as the nitrogen substituent throughout (Table 3).

Replacement of the succinimide moiety of thalidomide with either cyclopentanedione or lactam structure has

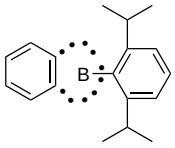
Table 2. Effects of Disubstituted *N*-Phenylphthalimides on TNF-α Production

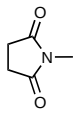
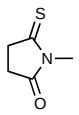
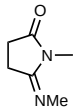
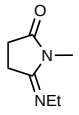


compd	code (30 μM)	R_1	R_2	amount of TNF-α (%)[a] TPA/HL-60	amount of TNF-α (%)[a] OA/HL-60	cytotoxicity[b] IC_{50} (μg/mL)
1	PP-00	H	H	100	61	>100
11	PP-11	2-Me	6-Me	161	40	>100
12	PP-0101	3-Me	5-Me	136	89	>100
13	PP-21	2-Et	6-Me	188	82	26.2 (99 μM)
14	PP-22	2-Et	6-Et	453	53	38.0 (136 μM)
15	PP-31	2-iPr	6-Me	234	33	28.1 (97 μM)
16	PP-32	2-iPr	6-Et	255	26	6.0 (20 μM)
17	PP-33	2-iPr	6-iPr	602	21	>100

[a,b] See Table 1.

Table 3. Effects of variation of the Succinimide Moiety of PP-33 on TNF-α Production Regulation

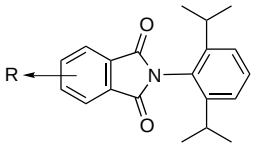


compd	B	code (30 μM)	amount of TNF-α (%)[a] TPA/HL-60	amount of TNF-α (%)[a] OA/HL-60	cytotoxicity[b] IC_{50} (μg/mL)
17		PP-33	602	21	>100
18		PPS-33	1252	0	30.8 (95 μM)
19		IP-33	214	40	43.1 (148 μM)
20		PIQ-33	268	50	>100
21		MIP-33	134	37	NT[c]
22		EIP-33	138	75	NT[c]

[a,b] See Table 1. [c] NT = not tested.

been reported to enhance the teratogenicity,[22] but the effect on TNF-α production has not been reported so far. Table 3 shows the effects of structural variation at the succinimide moiety of the potent TNF-α production regulator PP-33 (**17**). As shown in the table, all of the compounds (except PPS-33: **18**) showed TNF-α production-regulating activity weaker than that of PP-33 (**17**) (Figure 2a,b). The monothiocarbonyl derivative (PPS-33: **18**) showed activity superior to that of PP-33 (**17**) (Table 3). It enhanced TPA-induced TNF-α production

Table 4. Effects of Substituents at the Phthaloyl Moiety of PP-33 on TNF-α Production Regulation



compd	code (30 μM)	R	amount of TNF-α (%)[a] TPA/HL-60	amount of TNF-α (%)[a] OA/HL-60	cytotoxicity[b] IC_{50} (μg/mL)
17	PP-33	H	602	21	>100
23	4NPP-33	4-NO_2	792	54	93.1 (264 μM)
24	5NPP-33	5-NO_2	683	54	42.7 (121 μM)
25	4APP-33	4-NH_2	248	21	8.1 (25 μM)
26	5APP-33	5-NH_2	242	28	17.4 (53 μM)
27	4HPP-33	4-OH	239	64	>100
28	5HPP-33	5-OH	153	7	12.7 (39 μM)
29	NAP-33[c]		144	48	>100

[a,b] See Table 1. [c] NAP-33 = 2-(2,6-diisopropylphenyl)-1*H*-naphthoisoindole-1,3-dione.

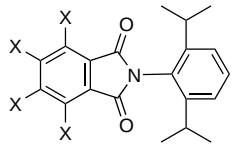
to more than 1200% and 1600% at 30 and 100 μM, respectively (Figure 2a), while it completely inhibited OA-induced TNF-α production at 30 μM (Figure 2b).

Effects of Substituents on the Condensed Benzene Ring. We next investigated the effects of substituents introduced at the condensed benzene ring of the phthalimide moiety (Table 4).[20] As shown in the table, introduction of an electron-donating group such as a hydroxyl (4HPP-33: **27**, 5HPP-33: **28**) or an amino group (4APP-33: **25**, 5APP-33: **26**) decreased the TPA-induced TNF-α production-enhancing activity (150−250%) compared to PP-33 (**17**, 602% in this experiment) (Table 4). The effects elicited by introduction of these two groups are similar. In both cases, the 4-substituted analogs (**25** and **27**) show higher activity than the corresponding 5-substituted analogs (**26** and **28**, respectively) (although **25** and **26** showed almost the same efficacy). In contrast to the introduction of electron-donating groups, introduction of the electron-withdrawing nitro group (4NPP-33: **23**, 5NPP-33: **24**) increased the enhancing activity (Table 4 and Figure 2a). In this case also, the TNF-α production-enhancing activity of the 4-nitro derivative (**23**, 792%) was higher than that of the 5-nitro derivative (**24**, 683%).

In contrast, introduction of the electron-withdrawing nitro group (**23**, **24**) decreased OA-induced TNF-α production-inhibiting activity (54%) compared to PP-33 (**17**, 21%). To our surprise, the electron-donating amino group (**25**, **26**) did not affect the activity (21−28%), and a 5-hydroxyl group (5HPP-33: **28**) enhanced the inhibiting activity (7%). It is curious that 4HPP-33 (**27**) showed less potent inhibiting activity (64%) than that of the nonsubstituted analogue PP-33 (**17**, 21%), despite the presence of the electron-donating hydroxyl group (Table 4). We speculated that a hydrogen bonding interaction between the 4-OH group and the neighboring imide carbonyl oxygen might be related to the decrease in inhibiting activity.

Effects of Halogenation of the Condensed Benzene Ring. It is of great interest that introduction of four fluorine atoms into the phthalimide moiety (FPP-33: **30**) greatly lowered the concentration of the compound which is necessary to elicit both TNF-α production-enhancing and -inhibiting activity (Table 5; the activity of FPP-33 (**30**) was assessed at 0.3 μM be-

Table 5. Effects of Halogenation at the Phthaloyl Moiety of PP-33 on TNF-α Production Regulation



compd	concn (μM)	code	X	amount of TNF-α (%)[a] TPA/ HL-60	amount of TNF-α (%)[a] OA/ HL-60	cytotoxicity[b] IC_{50} (μg/mL)
17	30	PP-33	H	602	21	>100
30	0.3	FPP-33	F	375	0	0.9 (2.3 μM)
31	30	ClPP-33	Cl	250	19	>100
32	30	BrPP-33	Br	235	3	NT[c]

[a] HL-60 cells were treated with 10 nM TPA or 50 nM OA in the presence of 30 μM (except FPP-33, 300 nM) test compound. The amount of TNF-α secreted in the culture medium was measured by ELISA. The amount of TNF-α production in the presence of 10 nM TPA alone or 50 nM OA alone was defined as 100%. [b] See Table 1. [c] NT = not tested.

cause it showed the maximum activity at this concentration, while the other compounds were assessed at 30 μM).[20] As previously reported, FPP-33 (**30**) exhibited TNF-α production-enhancing activity amounting to more than 300% at 0.1 μM (Figure 2a). On the other hand, the tetrachloro (ClPP-33: **31**) and tetrabromo (BrPP-33: **32**) analogues are much less active than FPP-33 (**30**), both as enhancers and inhibitors. Both ClPP-33 (**31**) and BrPP-33 (**32**) were less active than 0.3 μM FPP-33 (**30**) even at 100 times higher concentration (30 μM) (Table 5). These observations suggest that the increase in activity is specific to tetrafluorination of the phthaloyl group. Recently, Liu and co-workers also reported this unique effect of tetrafluorination.[23] They synthesized tetrafluorothalidomide and found that the compound is over 500-fold more potent than thalidomide. This promising effect with respect to TNF-α production regulation cannot be explained only in terms of the electronegative character of the fluorine substituents; the reasons remain unknown. The dose–response curves for the enhancing activity of the halogenated compounds on TNF-α production are shown in Figure 2a–c.

Because FPP-33 (**30**) showed potent TNF-α production-regulating activity, we attempted to assay the TNF-α production-regulating activity of the compound in normal human peripheral blood cells. However, the results were not reproducible: sometimes enhancement was observed and sometimes inhibition. Normal peripheral blood cells used in our experiments might be heterogeneous in their responses to cell stimulators and to our compounds. In relation to this phenomenon, we previously reported cell-type-dependent bidirectional-regulation of TNF-α production with our compounds.[17] As a cell stimulator/inducer of TNF-α production, lipopolysaccharide (LPS) is considered to play a central role in the pathogenesis of septic shock syndromes. Unfortunately, HL-60 is not induced by LPS to produce TNF-α protein. Liu and co-workers reported that one of our compounds, FPP-33 (**30**), strongly inhibited LPS-induced TNF-α production by vitamin D_3-treated THP-1 cells.[23] We also observed potent inhibitory activity of FPP-33 (**30**) on LPS-induced TNF-α production using a mouse macrophage-like cell line, J774.1,[24] with an IC_{50} of 250 nM (Figure 3).

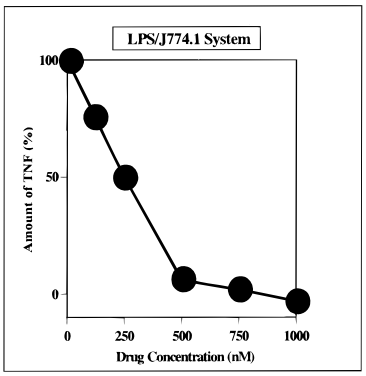


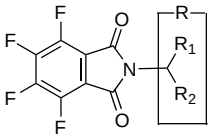
Figure 3. Effects of FPP-33 on LPS-induced TNF-α production inhibition. Horizontal scale: concentration of added test compound. Vertical scale: amount of TNF-α (%, vide infra). J774.1 cells were treated with 1 μg/mL of LPS in the presence of various concentrations of FPP-33. The amount of TNF-α secreted in the culture medium was measured by ELISA. The amount of TNF-α production in the presence 1 μg/mL of LPS alone was defined as 100%.

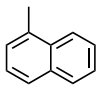
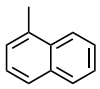
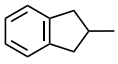
Structure–Activity Relationships of the Novel Series of *N*-Benzylphthalimides. As already reported, optically active phthalimide derivatives showed enantiodependent regulation of TNF-α production by HL-60 cells.[18] That is (*S*)-methylthalidomide (Figure 1), (*S*)-FPTP (**34**), and (*S*)-FPTH (**38**) (Table 6) exhibited an enhancing effect on TPA-induced TNF-α production by HL-60 cells, and the corresponding (*R*)-enantiomers ((*R*)-methylthalidomide (Figure 1), **33**, and **37** (Table 6), respectively) were completely inactive, while (*R*)-methylthalidomide, (*R*)-FPTP (**33**), and (*R*)-RPTH (**37**) showed more potent inhibiting activity than the corresponding (*S*)-enantiomers on OA-induced TNF-α production by the same cell line.

To examine the generality of this phenomenon and to study the structure–activity relationship of compounds of this type, further chemical modification of both enantiomers of FPTP (**33**, **34**) was performed. Because tetrafluorination dramatically lowered the effective concentration of the compounds as stated above, we focused on the tetrafluorophthalimide skeleton and assayed the compounds at 0.3 μM. The results are summarized in Tables 6 and 7. Elongation of the side chain methyl group of (*S*)-FPTP (**34**) to an ethyl group [(*S*)-FPEP (**40**)] enhanced the TPA-induced TNF-α production, but this modification greatly decreased OA-induced TNF-α production (Table 6). Cyclization of the side chain of (*S*)-FPTP (**34**) [(*S*)-FP13P (**42**)] greatly increased the TNF-α production-enhancing activity (Table 6). It is of interest that the antipodal (*R*)-enantiomer [(*R*)-FP13P (**41**)] also showed TNF-α production-enhancing activity. Cyclization of the side chain (**41**–**43**) decreased OA-induced TNF-α production (Table 6).

FP22P (**43**), a positional isomer of FP13P, lacked enhancing activity, but it showed moderate inhibiting activity. This means that not the length, but the width of the substituent introduced at the imide nitrogen is critical for potent TNF-α production-enhancing activity. In contrast, a sterically bulky substituent at the imide nitrogen is not necessary for potent TNF-α production-inhibiting activity. These results indicated a distinct structural requirement for the succinimide moiety of *N*-phenylphthalimide for potent TNF-α production enhancement, while the requirement in the case of TNF-α production inhibition was considerably less strict.

Table 6. Effects of N-Substituted Phthalimides on TNF-α Production

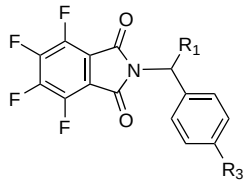


compd	code (0.3 μM)	R_1	R_2	amount of TNF-α (%)[a] TPA/ HL-60	OA/ HL-60	cytotoxicity[b] IC_{50} (μg/mL)
33	(*R*)-FPTP	CH_3		97	2	12.8 (40 μM)
34	(*S*)-FPTP	CH_3		143	70	26.8 (83 μM)
35	(*R*)-FPTN	CH_3		100	2	2.8 (8 μM)
36	(*S*)-FPTN	CH_3		101	101	2.8 (8 μM)
37	(*R*)-FPTH	CH_3		98	10	>100
38	(*S*)-FPTH	CH_3		124	65	>100
39	(*R*)-FPEP	C_2H_5		111	67	NT[c]
40	(*S*)-FPEP	C_2H_5		235	87	NT[c]
41	(*R*)-FP13P	R =		233	43	30.3 (87 μM)
42	(*S*)-FP13P	R =		392	98	>100
43	FP22P	R =		104	43	0.9 (2.6 μM)

[a] HL-60 cells were treated with 10 nM TPA or 50 nM OA in the presence of 0.3 μM test compound. The amount of TNF-α secreted in the cuture medium was measured by ELISA. The amount of TNF-α production in the presence of 10 nM TPA alone or 50 nM OA alone was defined as 100%. [b] See Table 1. [c] NT = not tested.

In the series of *N*-benzylphthalimide derivatives (**44**−**49**, Table 7), introduction of a substituent at the *para* position of the benzyl moiety enhanced TPA-induced TNF-α production. Compounds **44**−**49** listed in Table 7 showed TNF-α production-enhancing activity amounting to >200 at 0.3 μM, while the activities of the mother compounds (FPTPs: **33** and **34**) were 97% and 143%. In this case, both enantiomers exhibited equipotent enhancing activity (compounds **44**−**49**). The enhancing activity does not depend on the electronic effect of the substituent, since the compounds with the electron-withdrawing nitro group (**44**, **45**) or the electron-donating amino group (**46**, **47**) showed equipotent TPA-induced TNF-α production-enhancing activity. Introduction of a substituent at the *para* position of the benzyl moiety of (*R*)/(*S*)-FPTP (**44**−**49**) generally increased the enhancing activity on TPA-induced TNF-α production. On the other hand, introduction of a *para* substituent decreased the inhibiting activity on OA-induced TNF-α production for (*R*)-isomers (compared with (R)-FPTP:**33**) and increased it for (*S*)-isomers (except (*S*)-FPTP-93 (**49**), which showed almost the same activity as (*S*)-FPTP (compared with (*S*)-FPTP: **34**) (Table 7)). Therefore, the degree of enantiodependence was decreased by the introduction of substituents at the *para* position of the benzyl moiety of FPTP. To summarize the structure−activity relationships of the *N*-benzylphthalimide derivatives, elongation of the side chain, cyclization of the side chain, and introduction of substituents at the *para* position of the benzyl moiety enhanced the TPA-induced TNF-α production by HL-60 cells, while these structural modifications decreased the OA-induced TNF-α production-inhibiting activity of (R)-FPTP (**33**). The degree of enantiodependence was decreased by these structural modifications. The dose−response curves for the inhibiting activity of optically active phthalimide derivatives on TNF-α production are shown in Figure 2d.

Table 7. Effects of *N*-Benzylphthalimides on TNF-α Production



compd	code (0.3 μM)	R_1	R_3	amount of TNF-α (%)[a] TPA/ HL-60	OA/ HL-0
33	(*R*)-FPTP	CH_3	H	97	2
34	(*S*)-FPTP	CH_3	H	143	70
44	(*R*)-FPTP-00N	CH_3	NO_2	239	27
45	(*S*)-FPTP-00N	CH_3	NO_2	268	32
46	(*R*)-FPTP-00A	CH_3	NH_2	241	27
47	(*S*)-FPT-00A	CH_3	NH_2	262	38
48	(*R*)-FPTP-93	CH_3	$NHCOCH_3$	220	55
49	(*S*)-FPTP-93	CH_3	$NHCOCH_3$	268	80

[a] See Table 6.

Discussion

The TNF-α production-regulating activity and structure−activity relationships of N-substituted phthalimides were investigated. On the basis of our experiments using HL-60 stimulated with TPA or OA, the structural requirements for the TNF-α production-enhancing activity are the presence of a bulky, hydrophobic substituent at the imide nitrogen moiety, phthalimide, or monothiophthalimide structure, and the introduction of electron-withdrawing groups at the phthalimide moiety. In contrast, structural requirements for the TNF-α production-inhibiting activity are the presence of a bulky hydrophobic substituent at the imide nitrogen moiety, and the introduction of electron-donating groups at the phthalimide moiety. Phthalimide structure is not always required for potent TNF-α production-inhibiting activity. Substitution of the four hydrogen atoms at the phthalimide moiety with fluorine greatly decreased the concentration of the compound which is necessary to elicit TNF-α production-regulating activity.

Tetrafluorophthalimide derivatives show activity at concentrations of the order of 10^{-8} M. The presence of compounds which are effective at such low concentrations and the well-defined structutre−activity relationships suggests that there is a distinct specific target molecule(s) [binding protein(s)] of phthalimide-type TNF-α production regulators. Though the molecular mechanism(s) of these compounds eliciting inducer/cell type-specific bidirectional regulatory activity on TNF-α production is not clear at this stage, we previously proposed the existence of two distinct target molecules of the TNF-α production regulators, of which one plays a role in enhancement of TNF-α production and the other transmits the inhibitory effect on TNF-α production.[16] In fact, we have found (*R*)-form-specific and (*S*)-form-specific binding proteins in HL-60 cells, while the nature of these binding proteins is not clear at this stage (details will be published elsewhere). Of course, other explanations are possible. Our phthalimides, especially FPP-33 (**30**) (bidirectional TNF-α production-regulator), (R)-FPTN (**35**) (selective TNF-α production-inhibitor) and (S)-FP13P (**42**) (selective TNF-α production-enhancer) should be useful tools to investigate TNF-α production-regulatory mechanisms, and they also represent lead compounds for the development of superior thalidomidal BRMs targeting TNF-α.

Concerning the spectrum/specificity of the phthalimide derivatives described in this paper (compounds **1**−**9**, **11**−**49**), these compounds were found to inhibit TNF-α production in both TPA-induced and OA-induced THP-1 cells (details will be published elsewhere). Analysis of the difference between THP-1 and HL-60 cells at the molecular level, in relation to the different TNF-α production-regulatory responses to our compounds, is in progress.

In conclusion, though interpretation of the cell type specificity and inducer specificity of our compounds at the molecular level must await further investigation, we believe that several of these compounds, especially FPP-33 (*R*)-FPTP, and (*R*)-FPTN, have potential for development as novel BRMs to control TNF-α production. Pharmacological evaluation of these compounds is in progress.

Experimental Section

General. Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H-NMR spectra were measured in $CDCl_3$ with TMS and the solvent peak as internal standards, on a JEOL JMN-A500 spectrometer. Mass spectra (MS) were obtained on a JEOL JMS-HX110 spectrometer. Optical rotations were measured on a JASCO DIP-100 digital polarimeter. Column chromatography was carried out on Merck silica gel 60. Analytical thin-layer chromatography (TLC) was performed on Merck precoated silica gel $60F_{254}$ plates, and the compounds were visualized by UV illumination (254 nm) or by heating 150 °C after spraying with phosphomolybdic acid in ethanol. Elemental analysis was performed in the microanalytical laboratory of the Institute of Molecular and Cellular Biosciences, The University of Tokyo, or at Kyorin Pharmaceutical Co., Ltd.

Cells and Measurement of TNF-α. HL-60 cells were maintained as previously described.[20] The exponentially growing cells in RPMI1640 medium supplemented with 10% v/v fetal bovine serum (2.5×10^5 cells in 0.5 mL) were treated or not treated with TPA or OA for 16 h at 37 °C using 24-well multidish plates. For testing the effects of compounds, cells were treated with TPA (10 nM) or OA (50 nM) as an inducer in the presence or absence of a sample compound at the concentrations indicated in the tables. Then the number of cells was counted, the cellular morphology was checked under a microscope, and the cells were collected by centrifugation (2000 rpm × 10 min). The amount of TNF-α in the supernatant was measured by the use of a human TNF-α ELISA system (Amersham Co.) according to the supplier's protocol. The amount of TNF-α produced in the presence of inducer alone was defined as 100%.

J774.1 cell line was kindly provided by Dr. Nishijima and maintained in RPMI 1640 containing 10% FCS (GIBCO). The cells at a concentration of 5×10^5 cells/mL in RPMI1640 containing 10% FCS were cultured in the presence or absence of 1×10^{-6} g/mL of LPS at 37 °C for 24 h with or without a test compound. The amount of TNF-α in the supernatant was measured by the use of a mouse TNF-α ELISA system (Amersham Co.) according to the supplier's protocol. To assess the cell toxicity of the compounds, human embryonic lung fibroblast WI-38 cells (Dainippon Pharmaceutical Co.) were plated (5×10^3 cells/well) in 150 μL of RPMI1640 medium supplemented with 10% v/v FCS in 96-well microtiter plates. Various concentrations of test compounds in 50 μL of culture medium were added to the wells, and the plates were incubated at 37 °C for 48 h. After the incubation, viable cell number was assessed by WST-1 assay.[19] Briefly, WSR-1 solution (10 μL, 3.3 mg/mL) containing 1-methoxy PMS (70 μg/mL) was added to each well, and the plate was incubated at 37 °C for 2 h. Then the absorbance at 450 nm (red formazan) was measured.

Chemicals. Most of the compounds listed in Tables 1−6 were prepared (yield; 34%−98%) by condensation of phthalic anhydride (or its derivatives) with appropriate amines without any solvent at 160−180 °C for 2 h. The product was separated by silica gel column chromatography and recrystallized from an appropriate solvent(s).

Physicochemical properties of PP-00 (**1**), PP-10 (**2**), PP-n50 (**3**), PP-30 (**4**), PP-M0 (**6**), PP-N0 (**7**), PP-11 (**11**), PP-0101 (**12**), PP-21 (**13**), PP-22 (**14**), PP-31 (**16**), PP-33 (**17**), PPS-33 (**18**), 4NPP-33 (**23**), 5NPP-33 (**24**), 4APP-33 (**25**), 5APP-33 (**26**), 4HPP-33 (**27**), 5HPP-33 (**28**), FPP-33 (**30**), ClPP-33 (**31**), (R)-FPTP (**33**), (S)-FTP (**34**), (R)-FPTN (**35**), (S)-FPTN (**36**), (R)-FPTH (**37**) and (S)-FPTH (**38**) were described previously.[16−18,20]

2-(2-Biphenylyl)-1*H*-isoindole-1,3-dione (PP-ph0: 5) (yield, 72%): mp 163−164 °C (from *n*-hexane/ethyl acetate); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.20−7.34 (5H, m), 7.50−7.54 (4H, m), 7.70 (2H, dd, J = 5.50, 3.05 Hz), 7.81 (2H, dd, J = 5.50, 3.05 Hz); MS m/z 371 (M^+). Anal. ($C_{20}H_{13}NO_2$) C, H, N.

2-(2,6-Diisopropylphenyl)-1*H*-naphthoisoindole-1,3-dione (NAP-33: 29) (yield, 34%): mp 251−252.3 °C (from *n*-hexane/ethyl acetate); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.18 (12H, d, J = 6.84 Hz), 2.77 (2H, hept, J = 6.84 Hz), 7.32 (2H, d, J = 7.73 Hz), 7.48 (1H, t, J = 7.73 Hz), 7.72−7.76 (2H, m), 8.09−8.13 (2H, m), 8.49 (2H, s); MS m/z 357 (M^+). Anal. ($C_{24}H_{23}NO_2$) C, H, N.

(*R*)-2-[1-(4-Nitrophenyl)ethyl]-4,5,6,7-tetrafluoro-1*H*-isoindole-1,3-dione [(*R*)-FPTP-00N: 44] (yield, 98%): mp 158−160 °C (from *n*-hexane/ethyl acetate). $[\alpha]^{20}_D$ +40.4° (*c* 0.365, $CH_3CO_2C_2H_5$); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.95 (3H, d, J = 7.33 Hz), 5.60 (1H, q, J = 7.33 Hz), 7.65 (2H, d, J = 8.85 Hz), 8.21 (2H, d, J = 8.85 Hz); MS m/z 368 (M^+). Anal. ($C_{16}H_8F_4N_2O_4$) C, H, N.

(*S*)-2-[1-(4-Nitrophenyl)ethyl-4,5,6,7-tetrafluoro-1*H*-isoindole-1,3-dione [(*S*)-FPTP-00N: 45] (yield, 78%): mp 158−159.5 °C (from *n*-hexane/ethyl acetate); $[\alpha]^{20}_D$ −43.3° (*c* 0.21, $CH_3CO_2C_2H_5$); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.95 (3H, d, J = 7.33 Hz), 5.60 (1H, q, J = 7.33 Hz), 7.65 (2H, d, J = 8.85 Hz), 8.20 (2H, d, J = 8.85 Hz); MS m/z 368 (M^+). Anal. ($C_{16}H_8F_4N_2O_4$) C, H, N.

2-(2,6-Diisopropylphenyl)-3-(methylimino)-1-oxo-1*H*-isoindole (MIP-33: 21). A mixture of **18** (500 mg, 1.55 mmol), methylamine hydrochloride (190 mg, 1.63 mmol), ethanol (20 mL), and 0.1 N NaOH solution (15 mL) was refluxed for 10 h, and the reaction mixture was evaporated. The residue was purified by flash column chromatography on silica gel, eluting with CH_2Cl_2/methanol (20:1 v/v). The crude product was recrystallized from *n*-hexane/ethyl acetate to give 160 mg (yield, 32%) of colorless needles (5:1 mixture of regioisomers): mp 114−115 °C (from *n*-hexane/ethyl acetate); ^{1}H-NMR (500 MHz, $CDCl_3$) major isomer δ 1.13−1.58 (12H, m), 2.87 (2H, quint, J = 7.02 Hz), 3.71 (3H, s), 7.26−7.28 (2H,

m), 7.41−7.45 (1H, m), 7.71−7.78 (2H, m), 8.04 (1H, dd, J = 7.01, 0.9 Hz), 8.12 (1H, d, J = 7.32 Hz); minor isomer δ 1.13−1.58 (12H, m), 2.87 (2H, quint, J = 7.02 Hz), 2.90 (3H, s), 7.26−7.28 (2H, m), 7.41−7.45 (1H, m), 7.66 (1H, t, J = 7.60 Hz), 7.71−7.78 (2H, m), 7.91 (1H, dt, J = 7.01, 0.9 Hz); MS m/z 320 (M^+). Anal. $C_{21}H_{24}N_2O$) C, H, N.

2-(2,6-Diisopropylphenyl)-3-(ethylimino)-1-oxo-1*H*-isoindole (EIP-33: 22). A procedure similar to that described for **21**, utilizing **18**, base, and ethylamine hydrochloride, afforded **22** (5:1 mixture of regioisomers) (yield, 35%): mp 132−133 °C (from *n*-hexane/ethyl acetate); ^{1}H-NMR ($CDCl_3$) major isomer δ 1.09−1.19 (12H, m), 1.30 (3H, t, J = 7.02 Hz), 2.71 (2H, quint, J = 7.02 Hz), 4.04 (2H, quint, J = 7.32 Hz), 7.24−7.27 (2H, m), 7.41−7.45 (1H, m) 7.69−7.76 (2H, m), 8.04−8.05 (2H, m), 8.12 (1H, d, J = 7.32 Hz); minor isomer δ 1.09−1.19 (15H, m), 2.85 (2H, quint, J = 7.02 Hz), 3.03 (2H, quint, J = 7.32 Hz), 7.24−7.27 (2H, m), 7.41−7.45 (1H, m), 7.65 (1H, t, J = 7.02 Hz), 7.69−7.76 (1H, m), 7.90 (1H, d, J = 7.32 Hz), 7.96 (1H, d, J = 7.32 Hz); MS m/z 334 (M^+). Anal. ($C_{22}H_{26}N_2O$) C, H, N.

***(R)*-2-[1-(4-Aminophenyl)ethyl]-4,5,6,7-tetrafluoro-1*H*-isoindole-1,3-dione [(*R*)-FPTP-00A: 46].** A mixture of **44** (150 mg, 0.407 mmol), 10% Pd−C (50 mg), and ethyl acetate (100 mL) was hydrogenated for 1 h. The catalyst was removed by filtration, and the filtrate was evaporated. The residue was purified by flash column chromatography on silica gel, eluting with CH_2Cl_2/methanol (20:1 v/v). The crude product was recrystallized from *n*-hexane/ethyl acetate to give 100 mg of colorless powder (yield, 73%): mp 175−177 °C (from *n*-hexane/ethyl acetate); $[\alpha]^{20}_D$ +67.4° (c 0.161, $CH_3CO_2C_2H_5$); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.86 (3H, d, J = 7.32 Hz), 3.67 (2H, br s), 5.43 (1H, q, J = 7.32 Hz), 6.63 (2H, d, J = 8.54 Hz), 7.29 (2H, d, J = 8.54 Hz); MS m/z 338 (M^+). Anal. ($C_{16}H_{10}F_4N_2O_2$) C, H, N.

***(S)*-2-[1-(4-Aminophenyl)ethyl]-4,5,6,7-tetrafluoro-1*H*-isoindole-1,3-dione [(*S*)-FPTP-00A: 47].** A procedure similar to that described for **46**, utilizing **45**, 10% Pd−C, and ethyl acetate, afforded [(*S*)-FPTP-00A: **47**] (yield, 75%): mp 175−177 °C (from *n*-hexane/ethyl acetate); $[\alpha]^{20}_D$ −66.4° (c 0.135, $CH_3CO_2C_2H_5$); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.86 (3H, d, J = 7.32 Hz), 3.67 (2H, br s), 5.43 (1H, q, J = 7.32 Hz), 6.63 (2H, d, J = 8.54 Hz), 7.29 (2H, d, J = 8.54 Hz); MS m/z 338 (M^+). Anal. ($C_{16}H_{10}F_4N_2O_2$) C, H, N.

***(R)*-2-[1-[4-(Acetylamino)phenyl]ethyl]-4,5,6,7-tetrafluoro-1*H*-isoindole-1,3-dione [(*R*)-FPTP-93: 48].** Acetyl chloride (78.5 mg, 1.00 mmol) was added dropwise to a solution of 46 (338 mg, 1.00 mmol), triethylamine (101 mg, 1.00 mmol), and $CHCl_3$ (20 mL) at 0 °C. The solution was stirred overnight at room temperature, and the reaction was quenched by the addition of water (100 mL). The organic phase was washed with 0.1 N NaOH and 0.1 N HCl and then evaporated. The residue was purified by flash column chromatography on silica gel, eluting with CH_2Cl_2/methanol (20:1 v/v). The crude product was recrystallized from *n*-hexane/ethyl acetate to give 210 mg of yellow powder (yield, 55%): mp 140−143 °C (from *n*-hexane/ethyl acetate); $[\alpha]^{20}_D$ +47.8° (c 0.298, $CH_3CO_2C_2H_5$); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.89 (3H, d, J = 7.32 Hz), 2.16 (3H, s), 5.49 (1H, q, J = 7.32 Hz), 7.13 (1H, br s), 7.44−7.45 (4H, m); MS m/z 380 (M^+). Anal. ($C_{18}H_{12}F_4N_2O_3$) C, H, N.

***(S)*-2-[1-[4-(Acetylamino)phenyl]ethyl]-4,5,6,7-tetrafluoro-1*H*-isoindole-1,3-dione [(*S*)-FPTP-93: 49].** A procedure similar to that described for **48**, using **47**, base, and acetyl chloride, afforded **49** (yield, 60%): mp 140−143 °C (from *n*-hexane/ethyl acetate); $[\alpha]^{20}_D$ −45.3° (c 0.788, $CH_3CO_2C_2H_5$); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.88 (3H, d, J = 7.32 Hz), 2.15 (3H, s), 5.48 (1H, q, J = 7.32 Hz), 7.17 (1H, br s), 7.42−7.46 (4H, m); MS m/z 380 (M^+). Anal. ($C_{18}H_{12}F_4N_2O_3$) C, H, N.

References

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