# 5′-Substituted Thalidomide Analogs as Modulators of TNF-α☆

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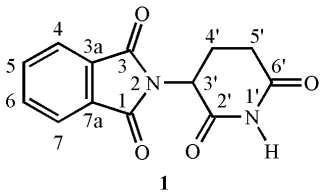
## Summary

The synthesis of 5′-substituted thalidomide analogs is described. The amino acids **2** necessary to synthesize the target compounds were prepared by Michael reaction. Condensation of **2** with phthalic anhydrides followed by reaction with urea yielded **4** as diastereomeric mixtures. Furthermore glutethimide (**5**) was brominated by an improved method and the resulting compound **6** was reacted in several steps with sodium azide, hydrogen, and phthalic anhydride to give **8**. In a similar manner, **6** was reacted with sodium azide and various phthalic anhydrides to give **9**, **10**, and **11**. All final compounds were tested *in vitro* for their inhibitory activity on the release of TNF-α, using stimulated peripheral mononuclear blood cells (PBMCs). Compounds with an additional aromatic substituent in position 5′ of the thalidomide molecule were more active than thalidomide. Compound **11** was able to reduce increased levels of IL-2 *in vitro*.

## Introduction

Thalidomide **1**, developed as a sedative, was introduced on the market in 1956. The drug quickly found broad acceptance because of its quality to induce a "physiological" sleep without the so called "hangover" syndrome which was sometimes observed with other sedatives such as derivatives of barbituric acids. Due to an apparent lack of severe toxicity, the drug was regarded as particularly safe. During this time, malformations were observed in newborn children which hitherto had occurred only in rare cases. Lenz [1] and McBride [2] were the first to suspect a link between these malformations and the use of thalidomide by pregnant women. Subsequently all preparations containing thalidomide were withdrawn from the market [1].



**1**

Thalidomide

However, some time later it was noted that the drug was very effective for treatment of erythema nodosum leprosum (ENL), an acute inflammatory manifestation of lepromatous leprosy[3]. Today, thalidomide is the drug of choice for treatment of this condition also known as type II leprosy reaction.

Besides teratogenicity and association with neuropathy, thalidomide was found to have some properties of pharmacological interest, e.g. it suppresses the release of tumor necrosis factor-α (TNF-α) produced by monocytes/macrophages [4] and it inhibits angiogenesis in animal models [5]. The immunomodulating and antiinflammatory properties of the drug are used in clinical studies in a variety of diseases including graft-*versus*-host-disease (GvHD) following bone marrow transplantation, rheumatoid arthritis, Behçet's disease, cachexia in AIDS, and several dermatological diseases [6–9]. It is supposed, that overexpression of the cytokine TNF-α plays a major role in many of these inflammatory manifestations. Substances which can effectively modulate the production of TNF-α may be of benefit for the treatment of disorders with excessive levels of TNF-α. Using thalidomide as a lead structure several analogs with structural modifications of the molecule have been described. *N*-substituted phthalimides with a simplified glutarimide moiety were reported by Hashimoto et al. Most of the synthesized compounds enhanced the release of TNF-α in vitro [10–13]. It was also found that the modulation of TNF-α release depends on the inducing agent, the used cell line and additionally on the optical properties of chiral compounds [14,15]. Muller et al. synthesized phthalimido β-amino acid derivatives as analogs of thalidomide which were effective in inhibition of TNF-α in vitro [16]. The influence of halogenation of the phthalimide moiety of thalidomide and several alkylated *N*-phenylphthalimide analogs was investigated by Liu et al. [17].

We also used thalidomide as lead structure and were especially interested in structural modifications at the position 5′ of the molecule. The synthesis of various derivatives with enhanced inhibition of release of TNF-α is described herein.

To our knowledge, only two 5′-substituted derivatives of thalidomide are described in the literature. The first one, 5′-hydroxy thalidomide could be a metabolite of thalidomide since it was found in traces after purification of a mixture of thalidomide with rat liver microsomes. The structure of 5′-hydroxy thalidomide was elucidated by chromatographic and spectroscopic methods [18]. The authors did not synthesize the compound therefore we synthesized the compound and evaluated its activity. The second 5′-substituted thalidomide analog is a derivative of glutethimide (**9**) [23] (see below).

## Chemistry

The 5′-monosubstituted thalidomide analogs **4** were prepared by conventional methods as shown in Scheme 1. The required substituted glutamic acids **2** were synthesized by Michael reaction of acetamidomalonate with α-substituted

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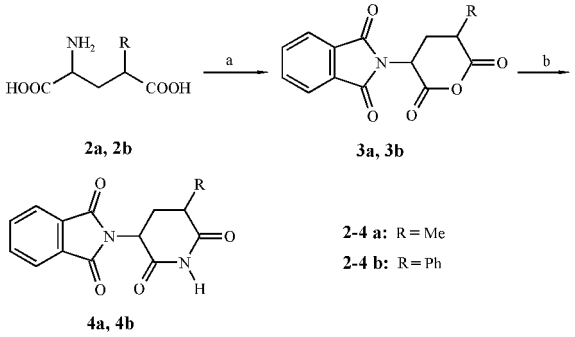
alkyl acrylates. Subsequent hydrolysis in concentrated hydrochloric acid gave the amino acids **2** in good yields as diastereomeric mixtures, which were used without further separation. γ-Phenylglutamic acid **2b** [19] was first synthesized by this method.




**Scheme 1.** Reagents: (a) 1. phthalic anhydride, pyridine, reflux, 2. acetic anhydride, reflux; (b) urea, 200 °C.

Phthalic anhydride was condensed with **2** in the presence of pyridine and, after elimination of the solvent, treated with acetic anhydride to give the anhydrides **3**. Final conversion of the anhydrides **3** to the imides **4** was accomplished by treating with urea at about 200 °C. The products were obtained as diasteromeric mixtures in a ratio of about 4:1 (**4a**) and about 6:1 (**4b**). The strong biological activity of **4b** (see below) prompted us to elucidate the relative configuration. Compound **4b** was tested as a mixture of diastereomers and it was assumed that the major diastereomer of **4b** was responsible for the inhibitory activity. The $^1$H-NMR data proved the cis-configuration (*R*,*R*;*S*,*S*) of the substituents at position 3′ and 5′ and a diequatorial conformation in the glutarimide moiety ( Figure 1).

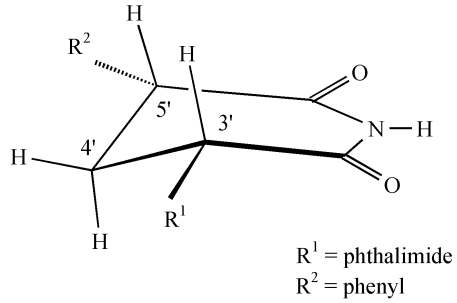
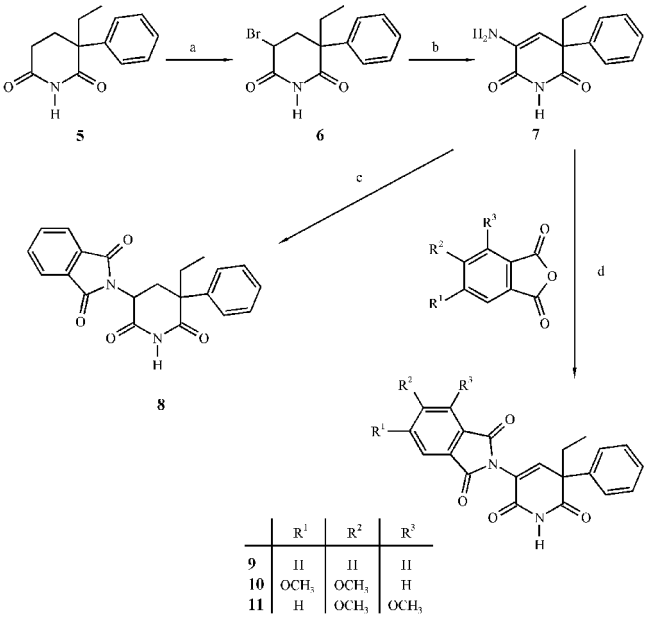



**Figure 1.** Conformation of the major diastereomer of **4b** (*rac*-cis).

It was assumed that the diequatorial position of the substituents bears importance for the inhibitory activity. To stabilize the substituents in such a position we decided to establish a double bond between C-3′ and C-4′ of the molecule. The easily available glutethimide (**5**) was utilized as educt. The synthetic route started with the bromination of **5** (Scheme 2).

The known procedures [20,21] to prepare **6** were improved by treatment of **5** with bromine in refluxing acetic acid. Further reaction of **6** with sodium azide in hot aqueous DMSO according to ref. [22] yielded **7**, which was utilized to prepare

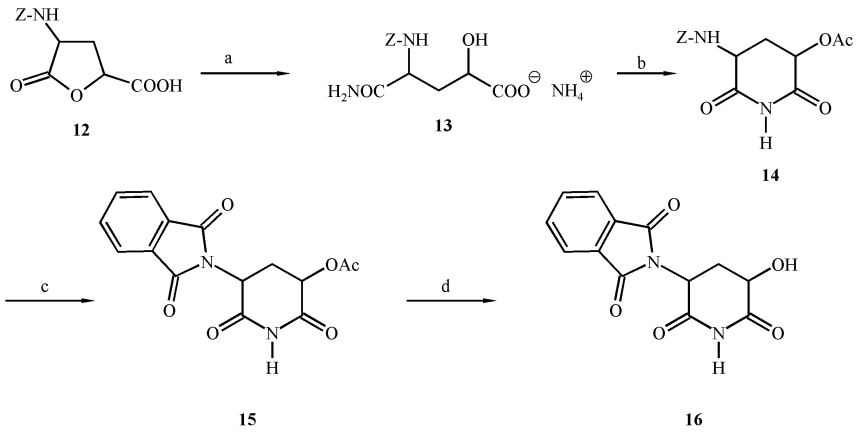



**Scheme 2.** Reagents: (a) $Br_2$, HOAc, reflux; (b) $NaN_3$, DMSO, $H_2O$, 100 °C; (c) 1. $H_2$, 10% Pd/C, EtOH, 2. phthalic anhydride, HOAc, reflux , (d) HOAc, reflux.

the saturated compound **8** and the desired unsaturated derivatives **9**, **10**, and **11**. Catalytic hydrogenation of **7** using palladium/charcoal followed by treatment with phthalic anhydride in acetic acid yielded **8**. The $^1$H-NMR data of **8** indicated the existence of only one diastereomer. It was assumed to be the cis-configuration (*rac*-cis) comparable to the major diastereomer of compound **4b** (*rac*-cis). To elucidate the correct relative configuration of the substance, further analyses using chromatographic methods are in preparation. Some reports in the literature showed the nitrogen of the amino group of **7** to be sufficiently nucleophilic to react with several electrophilic substances [21,23]. For instance **7** was treated by Casini et al. [23] with phthalic anhydride at high temperatures without solvent to give **9**. We found an improved method by reacting **7** with various phthalic anhydrides in refluxing acetic acid. The products obtained (**9**, **10**, and **11**) crystallized spontaneously in high purity after cooling of the reaction mixtures.

To investigate the influence of the polar hydroxy group in position 5′ of the thalidomide molecule 5′-hydroxy thalidomide **16** was synthesized as shown in Scheme 3.

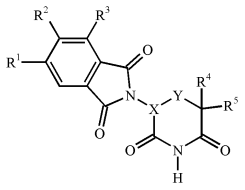
We started with the preparation of γ-hydroxyglutamic acid as described [24]. From the mixture of the diastereomers, the threo-form (*rac*-trans) was separated as the protected lactone **12** [25]. Subsequent synthetic steps were carried out only with this threo-form. **12** was reacted with a saturated solution of dry ammonia in methanol to give **13** which was cyclized to **14** as nearly a single diastereomer (*rac*-trans **14**). Deprotection of the amino group followed by reaction with phthalic anhydride in acetic acid yielded **15** as nearly a single diastereomer (*rac*-cis **15**) together with a mixture of diastereomers in a ratio of about 1:2. The single diastereomer **15** (*rac*-cis) was subsequently heated with p-toluenesulfonic acid in dry methanol to give **16** (*rac*-cis). The relative configuration of **16** was elucidated by NMR experiments.



**Scheme 3.** Reagents:(a) ammonia, methanol, (b) acetic anhydride, reflux, (c) 1. $H_2$/10% Pd/C, ethanol, 2. phthalic anhydride, acetic acid, reflux, (d) p-toluenesulfonic acid, methanol, reflux.

All tested compounds were substituted in position 5′ of the original thalidomide molecule. Substitution of one proton by a methyl group (**4a**) decreased slightly the inhibitory activity. On the other hand, the introduction of a polar hydroxy group in the glutarimide moiety (**16**) decreased the activity compared to thalidomide. It can be concluded that the metabolic hydroxylation in position 5′ of the molecule had no influence on the release of TNF-α by thalidomide. Compared with **16**, acetylation of the hydroxy group (compound **15**) improved the activity only slightly. 5′-Phenyl thalidomide **4b** was found to be 5-fold more active than thalidomide in inhibition of TNF-α release. Since **4a** and **4b** have two chiral carbon atoms they can occur in four stereoisomers in form of two diastereomers. The ratio between the diastereomers possibly depends on the steric properties of the substituents. In every case, we isolated a mixture of diastereomers with an excess of one diastereomer. In compound **4b** the diastereomeric excess was slightly higher than in **4a**. We assume, that the major diastereomer (*rac*-cis) of **4b** was responsible for the strong activity. The preferred conformation of this diastereomer could be important for the biological activity. The separation of the major diastereomer to examine the inhibitory activity is under way.

**Table 1.** TNF-α inhibition by 5′-substituted thalidomide analogs.



| No. | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | X-Y | Inhibition of release of TNF-α Value (%) at 50 μg/ml | $IC_{50}$ (μM) |
|---|---|---|---|---|---|---|---|---|
| **1** | H | H | H | H | H | CH–$CH_2$ | 65 | 39–58 |
| **4a** | H | H | H | H | Me | CH–$CH_2$ | 48 | n.d.[a] |
| **4b** | H | H | H | H | Ph | CH–$CH_2$ | 69 | 8.7 |
| **8** | H | H | H | Et | Ph | CH–$CH_2$ | 80 | 11.0 |
| **9** | H | H | H | Et | Ph | C=CH | 42 | n.d. |
| **10** | OMe | OMe | H | Et | Ph | C=CH | 0 | n.d. |
| **11** | H | OMe | OMe | Et | Ph | C=CH | 90 | 2.0 |
| **15** | H | H | H | H | OAc | CH–$CH_2$ | 36 | n.d. |
| **16** | H | H | H | H | OH | CH–$CH_2$ | 22 | n.d. |

[a] n.d.: not determined.

## Results and Discussion

Inhibition of release of TNF-α was measured in the supernatant of human PBMCs stimulated with LPS. All substances were screened in the first line using an initial concentration of 50 μg/ml. Compounds **4a** and **4b** were tested as diastereomeric mixtures. If a compound showed a better inhibitory activity than thalidomide, the $IC_{50}$ values were determined by using different concentrations of the substance. The results are shown in Table 1.

Compound **8** has an additional ethyl and phenyl group located at the position 5′ of the original thalidomide molecule. The $IC_{50}$ of 11 μM is comparable to compound **4b**. It is therefore to be concluded that an additional aromatic system in position 5′ of thalidomide enhances the inhibitory activity. As a result of the introduction of a $\Delta^3$ double bond in compounds **9, 10,** and **11** the hybridization of the carbon atom at position 3′ has changed from $sp^3$ to $sp^2$. At the same time, the molecules are less flexible in the glutarimide moiety and, additionally, the compounds have only one chiral center in comparison to **4a** and **4b**, which occur as diastereomeric mixtures. **9** without any substitution at the phthalimide ring enhanced the release of TNF-α. **10** is symmetrically substituted by two methoxy groups in position 5 and 6 of the phthalimide moiety and it showed no inhibitory activity. A small change in the substitution pattern by the two methoxy groups in **11**, showed a dramatic increase of the activity. **11** was found to be nearly 25-fold more active than thalidomide with a $IC_{50}$ of 2 μM. Obviously, the inhibitory activity of the prepared glutethimide derivatives **9**–**11** depends strongly on the substitution pattern in the phthalimide moiety.

Compound **11** was also found to inhibit the release of IL-2 *in vitro*. Human PBMCs were stimulated by monoclonal antibodies CD2/CD28 and staphylococcal TSST-1. At a concentration of 119 μM, **11** decreased the concentration of IL-2 by 86±6% (CD2/CD28) and 77±20% (TSST-1). In this assay thalidomide showed only weak activity.

## Conclusions

Using thalidomide as a lead structure, we have prepared new analogs which inhibit TNF-α production to varying degrees in LPS-stimulated human PBMCs. All synthesized compounds were substituted in position 5′ of the original thalidomide molecule. Only compounds with an aromatic substitution showed enhanced inhibitory activity on the release of TNF-α by stimulated PBMCs. The ability of compound **11** to decrease enhanced IL-2 concentrations can be of value with regard to the role of this cytokine in inflammation and immune response.

## Acknowledgment

Thanks are due to the Verband der Chemischen Industrie, Fonds der Chemischen Industrie for supporting this work.

## Experimental section

Melting points were determined on a Boetius melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer FT-IR PC 16 spectrophotometer and NMR data were obtained with a Varian Gemini 300 spectrometer using tetramethylsilane as an internal standard. The IR and NMR spectra of each compound were consistent with the assigned structure. Elemental analysis were performed at the Institute of Organic Chemistry, University Leipzig and were within ± 0.4 % of the theoretical values. Mass spectra were obtained on a Hewlett Packard LC/MS (LC: HP 1050, MS: HP-MS-Engine 5989 A). Thin layer chromatography was performed using silica gel 60 $F_{254}$ (Merck). Solvents were dried by conventional methods. α-Methylglutamic acid **2a** was synthesized according to ref.[26], diethyl acetamidomalonate was used as reactant.

*γ-Phenylglutamic acid (Diastereomeric mixture) (**2b**)*

A suspension of 18.90 g (87.10 mmol) diethyl acetamidomalonate in a solution of 0.20 g (8.70 mmol) of sodium in 50 ml of ethanol was stirred for 15 min. A solution of 17.00 g (91.60 mmol) of ethyl α-phenylacrylate in 20 ml of ethanol was added dropwise to the orange coloured suspension to keep the mixture gently boiling. After 2.5 h the reaction was stopped with 5 ml glacial acetic acid and the solution was evaporated to dryness. The residue was refluxed in 80 ml of concentrated hydrochloric acid for 16 h. After cooling, the solution was extracted with 50 ml of ethyl acetate and the aqueous layer was evaporated to dryness. The residue was coevaporated three times with 100 ml of water and then dissolved in 50 ml of water. The solution was adjusted to pH 3 with ammonia solution (25%). The precipitated product was filtered off and washed with 200 ml of ice cold water to yield 10.80 g (51%) as raw product which was used without further purification.

Recrystallisation from water yielded a single diastereomer with mp 220–223 °C.– FT-IR(KBr) ν = 3538, 2938, 1696, 1624, 1600, 1532, 1446, 1358, 1292, 1252.– $^1$H-NMR δ = 2.03–2.27 (m, 2H, $CH_2$), 2.99–3.04 (m, 1H, 4-H), 3.60–3.65 (m, 1H, 2-H), 7.35–7.44 (m, 5H, aromatic H).– $^{13}$C-NMR δ = 39.02 (C-3), 51.92 (C-4), 54.62 (C-2), 127.03, 128.30, 128.92, 141.08 (C-aromatic), 182.28, 183.39 (C=O).– Anal. ($C_{14}H_{11}NO_5 \bullet H_2O$)

No attempt was made to isolate the other diastereomer.

*2-(5-Methyl-2,6-dioxo-tetrahydro-pyran-3-yl)-1H-isoindole-1,3[2H]-dione (**3a**)*

A mixture of 2.00 g (11.20 mmol) of **2a** and 1.95 g (13.20 mmol) of phthalic anhydride was refluxed in 15 ml of pyridine for 6 h. The solvent was removed in vacuo and the residue was refluxed in acetic anhydride for 1 h. After cooling, the precipitated product was collected by filtration and washed with 50 ml of ether. A second fraction was obtained by evaporation of the mother liquor to dryness and dilution of the residue with 20 ml of ether. The combined crude products were recrystallized from toluene to yield 2.00 g (65%) with mp 223–225 °C.– FT-IR (KBr) ν = 1814, 1764, 1712, 1394, 1090, 1030.– $^1$H-NMR δ = 1.27 (d, *J* = 6.8 Hz, 3H, $CH_3$), 2.15–2.48 (m, 2H, 4′-H), 3.28–3.33 (m, 1H, 5′-H), 5.54 (dd, *J* = 13.0, 5.7 Hz, 1H, 3′-H), 7.89–7.97 (m, 4H, aromatic H).– $^{13}$C-NMR δ = 15.12 ($CH_3$), 28.15 (C-4′), 35.07 (C-5′), 48.26 (C-3′), 123.61, 131.04, 135.04 (C-aromatic), 165.74, 166.59, 169.29 (C=O).– Anal. ($C_{14}H_{11}NO_5$)

*2-(5-Phenyl-2,6-dioxo-tetrahydro-pyran-3-yl)-1H-isoindole-1,3[2H]-dione (**3b**)*

A mixture of 3.00 g (12.40 mmol) of **2b** and 2.10 g (14.30 mmol) of phthalic anhydride was refluxed in 40 ml of pyridine for 6 h. The hot solution was filtered and evaporated in vacuo to dryness. The residue was dissolved in 50 ml of hydrochloric acid (5%) and extracted three times with 50 ml of ethyl acetate. The combined organic layers were washed with water and dried over sodium sulfate. The solvent was evaporated in vacuo and the residue was refluxed in 40 ml of acetic anhydride for 1 h. The solvent was removed in vacuo to a residue of about 5 ml which was diluted with 30 ml of ether. The precipitated product was filtered off, washed with 100 ml of ether and recrystallized from toluene to yield 2.60 g (63%) with mp 163–165 °C.– (FT-IR) ν = 1816, 1774, 1716, 1392, 1040.– $^1$H-NMR (major diastereomer) δ = 2.34–2.38 (m, 1H, 4′-H), 2.93–2.98 (m, 1H, 4′H), 4.64 (dd, *J*= 13.7 , 4.9 Hz, 1H, 5′-H), 5.73 (dd, *J* = 13.1 , 5.4 Hz, 1H, 3′-H), 7.33–7.38 (m, 5H, aromatic H phenyl), 7.89–7.94 (m, 4H, aromatic H phthalimide).– $^{13}$C-NMR (major diastereomer) δ = 28.61 (C-4′), 46.55 (C-5′), 48.45 (C-3′), 123.64, 131.07, 135.07 (C-aromatic phthalimide), 127.73, 128.58, 128.67, 136.68 (C-aromatic phenyl), 165.38, 166.58, 167.49 (C=O).– Anal. ($C_{19}H_{13}NO_5$)

*2-(5-Methyl-2,6-dioxo-piperidin-3-yl)-1H-isoindole-1,3[2H]-dione (**4a**)*

A mixture of 2.00 g (7.30 mmol) of **3a** and 0.23 g (3.80 mmol) of dried urea was heated to 185–190 °C for 30 min. After cooling, the residue was first refluxed in 5 ml of acetic anhydride for 5 min. Then 5 ml of ethanol was added to the suspension at about 80 °C and the mixture was refluxed for 10 min. The precipitated product was filtered off and recrystallized from DMF/$H_2O$ to yield 1.35 g (68%) as diastereomeric mixture with mp 270–272 °C.– (FT-IR) ν = 1778, 1728, 1386, 1228.– $^1$H-NMR (major diastereomer) δ = 1.16 (d, *J* = 6.8 Hz, 3H, $CH_3$), 2.11–2.16 (m, 1H, 4′-H), 2.28–2.41 (m, 1H, 4′-H), 2.93–3.02 (m, 1H, 5′-H), 5.24 (dd, *J* = 12.9, 5.2 Hz, 1H, 3′-H), 7.89–7.94 (m, 4H, aromatic-H), 11.11 (s, 1H, NH).– $^{13}$C-NMR (major diastereomer) δ = 14.80 ($CH_3$), 30.08 (C-4′), 35.39 (C-5′), 49.40 (C-3′), 123.25, 131.18, 134.85(C-aromatic), 167.11, 170.02, 175.21 (C=O).– MS(EI); *m/z* = 258 (30) $[M]^+$.– Anal. ($C_{14}H_{12}N_2O_4$)

*2-(5-Phenyl-2,6-dioxo--piperidin-3-yl)-1H-isoindole-1,3[2H]-dione (**4b**)*

The procedure followed the synthesis of **4a**. From 2.00 g (6.00 mmol) of **3b** was yielded 0.80 g (40%) **4b** as diastereomeric mixture with mp 228–231 °C.– (FT-IR) ν = 3088, 1780, 1760, 1392, 1220.– $^1$H-NMR (major diastereomer) δ = 2.25–2.89 (m, 2H, 4′-H), 4.34 (dd, *J* = 13.6 , 4.7 Hz, 1H, 5′-H), 5.40 (dd, *J* = 12.9, 5.2 Hz, 1H, 3′-H), 7.22–7.39 (m, 5H, aromatic H phenyl), 7.87–7.79 (m, 4H, aromatic H phthalimide), 11.35 (s, 1H, NH).– $^{13}$C-NMR (major diastereomer) δ = 30.39 (C-4′), 47.25 (C-5′), 49.51 (C-3′), 123.27, 123.58, 131.20, 134.84, 134.92 (C-aromatic phthalimide), 127.11, 128.43, 128.68, 138.25 (C-aromatic phenyl), 166.99, 167.17, 169.76, 173.46 (C=O).– MS(EI); *m/z* = 334 (57) $[M]^+$.– Anal. ($C_{19}H_{14}N_2O_4$)

*2-(5-Ethyl-5-phenyl-2,6-dioxo-piperidin-3-yl)-1H-isoindole-1,3[2H]-dione (**8**)*

The solution of 1.00 g (4.30 mmol) of **7** in 40 ml of ethanol was vigorously stirred under a atmosphere of hydrogen in the presence of 0.10 g of palladium/charcoal (10%) until the starting material disappeared (TLC). The catalyst was filtered off and the solution evaporated to dryness. The residue was refluxed with 0.70 g (4.80 mmol) of phthalic anhydride in 40 ml of glacial acetic acid for 4 h. After cooling, the solvent was removed in vacuo and the residue was recrystallized from ethanol to yield 0.99 g (63%) with mp 174–177 °C.– (FT-IR) ν = 3226, 1720, 1668, 1392.– $^1$H-NMR δ = 0.76 (t, *J* = 7.3 Hz, 3H, $CH_2CH_3$), 1.85–1.95 (m, 2H, $CH_2CH_3$), 2.75–2.88 (m, 2H, 4′-H), 4.59 (dd, *J* = 13.1, 4.9 Hz, 1H, 3′-H), 7.37–7.51 (m, 5H, aromatic H phenyl), 7.89–7.92 (m, 4H, aromatic H phthalimide), 11.55 (s, 1H, NH).– $^{13}$C-NMR δ = 8.86 ($CH_3$), 30.24 (C-4′), 32.66 ($CH_2$), 47.22 (C-3′), 51.74 (C-5′), 123.37, 123.61, 131.04, 131.07, 134.95, 135.03 (C-aromatic

phthalimide), 125.87, 127.78, 129.18, 138.73 (C-aromatic phenyl), 167.02, 168.87, 174.59 (C=O).– MS(EI); *m/z* = 362 (43) $[M]^+$.– Anal. ($C_{21}H_{18}N_2O_4$)

*2-(5-Ethyl-5-phenyl-2,6-dioxo-1,2,5,6-tetrahydro-pyridin-3-yl)-1H-isoindole-1,3[2H]-dione (**9**)*

A mixture of 0.50 g (2.20 mmol) of **7** and 0.40 g (2.70 mmol) of phthalic anhydride was refluxed in 15 ml of glacial acetic acid for 6 h. After cooling the precipitated product was collected by filtration, washed with 20 ml glacial acetic acid and 20 ml of ether to give after drying in vacuo an analytically pure product. Yield was 0.62 g (78%) with mp 273–274 °C (ref.[15] 275–276.5 °C) (FT-IR) ν = 3186, 3072, 1724, 1690, 1662, 1378, 1238.– $^1$H-NMR δ = 0.92 (t, *J* = 7.2 Hz, 3H, $CH_2CH_3$), 2.03–2.10 (m, 1H, $CH_2CH_3$), 2.55–2.62 (m, 1H, $CH_2CH_3$), 7.36–7.54 (m, 6H, aromatic H phenyl, 4′-H), 7.93–8.02 (m, 4H, aromatic H phthalimide), 11.72 (m, 1H, NH).– $^{13}$C-NMR δ = 9.34 ($CH_3$), 30.10 ($CH_2$), 55.02 (C-5′), 122.83 (C-3′), 123.78, 131.28, 135.16 (C-aromatic phthalimide), 126.83, 128.15, 128.91, 138.91 (C-aromatic phenyl), 150.51 (C-4′), 161.50, 166.33, 166.58, 173.71 (C=O).– MS(EI); *m/z* = 360 (28) $[M]^+$.– Anal. ($C_{21}H_{16}N_2O_4$)

*2-(5-Ethyl-5-phenyl-2,6-dioxo-1,2,5,6-tetrahydro-pyridin-3-yl)-5,6-dimethoxy-1H-isoindole-1,3[2H]-dione (**10**)*

Preparation followed the synthesis of **9**, from 0.45 g (1.90 mmol) of **7** and 0.45 g (2.10 mmol) of 4,5-dimethoxyphthalic anhydride in a yield of 0.54 g (65%) with mp 288–290 °C.– (FT-IR) ν = 3436, 1716, 1372, 1310, 1224.– $^1$H-NMR δ 0.95 (t, *J* = 7.4 Hz, 3H, $CH_2CH_3$), 2.06–2.11 (m, 1H, $CH_2CH_3$), 2.51–2.59 (m, 1H, $CH_2CH_3$), 3.96 (s, 6H, $OCH_3$), 7.44–7.54 (m, 8H, aromatic H, 4′-H), 11.61 (s, 1H, NH).– $^{13}$C-NMR δ = 9.31 ($CH_3$), 30.20 ($CH_2$), 55.06 (C-5′), 56.54 ($OCH_3$), 106.15, 124.69, 154.22 (C-aromatic phthalimide),123.15 (C-3′), 126.84, 128.06, 128.85, 139.08 (C-aromatic phenyl), 150.14 (C-4′), 161.57, 166.58, 173.68 (C=O).– MS(EI); *m/z* = 420 (51) $[M]^+$.– Anal. ($C_{23}H_{20}N_2O_6$)

*2-(5-Ethyl-5-phenyl-2,6-dioxo-1,2,5,6-tetrahydro-pyridin-3-yl)-4,5-dimethoxy-1H-isoindole-1,3[2H]-dione (**11**)*

Preparation followed the synthesis of **9** using 0.45 g (1.90 mmol) **7** and 0.45 g (2.10 mmol) of 3,4-dimethoxyphthalic anhydride. After recrystallisation from ethanol 0.55 g (67%) of **11** was yielded with mp 203–205 °C.– (FT-IR) ν = 3218, 1724, 1496, 1374, 1276.– $^1$H-NMR δ = 0.91–0.97 (m, 3H, $CH_2CH_3$), 2.07–2.09 (m, 1H, $CH_2CH_3$), 2.57–2.59 (m, 1H, $CH_2CH_3$), 3.95 (s, 3H, $OCH_3$), 3.99 (s, 3H, $OCH_3$), 7.31–7.70 (m, 8H, aromatic H, 4′-H), 11.69 (s, 1H, NH).– $^{13}$C-NMR δ = 9.34 ($CH_3$), 29.98 ($CH_2$), 55.02 (C-5′), 56.75 ($OCH_3$), 61.85 ($OCH_3$), 117.72, 120.29, 122.99, 123.36, 146.73, 158.02 (C-aromatic phthalimide), 121.78 (C-3′), 126.83, 128.12, 128.90, 138.95 (C-aromatic phenyl), 150.42 (C-4′) 161.54, 164.44, 165.73, 173.74 (C=O).– MS(EI); *m/z* = 420 (15) $[M]^+$.– Anal. ($C_{23}H_{20}N_2O_6$)

*Acetic acid 5-benzyloxycarbonylamino-2,6-dioxo-piperidin-3-yl ester (**14**)*

A solution of 10.00 g (31.90 mmol) of **13** [17] was refluxed in 100 ml of acetic anhydride for 3 h. The solution was evaporated to dryness. The residue was dissolved in 10 ml of ethanol and diluted with 200 ml of water. The precipitated product was filtered off and recrystallized from $H_2O$ to yield 7.50 g (73%) with mp 149–150 °C.– (FT-IR) ν = 3332, 1746, 1728, 1260.– $^1$H-NMR δ = 2.10 (s, 3H, $CH_3$), 2.21–2.28 (m, 2H, 4-H), 4.38–4.42 (m, 1H, 3-H), 5.07 (s, 2H, $CH_2O$), 5.54 (dd, *J* = 6.9 , 5.6 Hz, 1H, 5-H), 7.31–7.37 (m, 5H, aromatic H), 7.97 (d, *J* = 8.5 Hz, 1H, CONH), 11.27 (s, 1H, 1-H).– $^{13}$C-NMR δ 20.46 ($CH_3$), 30.09 (C-4), 48.20 (C-3), 65.83 ($CH_2O$), 66.72 (C-5), 127.81, 127.88, 128.35, 136.70 (C-aromatic), 156.09 (CONH), 168.90, 169.22, 170.75 (C=O).– MS(TSP); *m/z* = 338 (64) $[M+18]^+$, 353 (100) $[M+33]^+$.– Anal. ($C_{15}H_{16}N_2O_6$)

*Acetic acid 5-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-2,6-dioxo-piperidin-3-yl ester (**15**)*

A solution of 3.00 g (9.40 mmol) of **14** was dissolved in 40 ml of glacial acetic acid and vigorously stirred in the presence of 0.30 g of palladium/charcoal (10%) under a hydrogen atmosphere for 3 h. The catalyst was filtered off and the resulting solution was refluxed with 2.08 g (14.00 mmol) of phthalic anhydride for 6.5 h. After cooling, the dark solution was stirred over night at room temperature. The precipitated product was collected by filtration and resolved in acetone. Addition of petroleum ether (bp 68–80 °C) yielded 1.20 g (40%) as single diastereomer with mp 253–256 °C. From the mother liquor 0.62 g (21%) was isolated as mixture of diastereomers.– (FT-IR) ν = 3216, 3100, 1720, 1394, 1224.– $^1$H-NMR (single diastereomer) δ = 2.12 (s, 3H, $CH_3$), 2.38–2.43 (m, 1H, 4-H), 2.70–2.75 (m, 1H, 4-H), 5.49 (dd, *J* = 13.1 , 5.4 Hz, 1H, 5-H), 5.86 (dd, *J* = 13.0 , 5.7 Hz, 1H, 3-H), 7.91 (s, 4H, aromatic H), 11.54 (s, 1H, NH).– $^{13}$C-NMR (single diastereomer) δ = 20.44 ($CH_3$), 27.60 (C-4), 47.69 (C-5), 67.37 (C-3), 123.40, 131.15, 134.95 (C-aromatic), 166.37, 167.11, 169.17, 169.90 (C=O).– MS (TSP); *m/z* = 334 (100) $[M+18]^+$.– Anal. ($C_{15}H_{12}N_2O_4$)

*2-(5-Hydroxy-2,6-dioxo-piperidin-3-yl)-1H-isoindole-1,3[2H]-dione (**16**)*

A solution of 1.00 g (3.20 mmol) of **15** (single diastereomer) and 0.30 g (1.60 mmol) of p-toluenesulfonic acid was refluxed in 30 ml of methanol for 5 h. After cooling, the precipitated product was filtered off and recrystallized from acetone/petroleum ether (bp 60–80 °C) or acetonitrile to yield 0.52 g (60%) with mp 195–230 °C.– (FT-IR) ν = 3410, 1786, 1394, 1224.– $^1$H-NMR δ = 2.27–2.53 (m, 2H, 4′-H), 4.53–4.57 (m, 1H, 5′-H), 5.29 (dd, *J* = 13.1, 5.2 Hz, 1H, 3′-H), 5.82 (d, *J* = 6.0 Hz, 1H, OH), 7.90–7.94 (m, 4H, aromatic H), 11.22 (s, 1H, NH).– $^{13}$C-NMR δ = 31.03 (C-4′), 48.22 (C-3′), 66.33 (C-5′), 123.29, 131.19, 138.88 (C-aromatic), 166.86, 176.17, 169.70, 174.71 (C=O).– MS(EI); *m/z* = 274 (13) $[M]^+$.– Anal. ($C_{13}H_{10}N_2O_5$)

*Assay for inhibition of TNF-α synthesis by human PBMCs*

PBMCs isolated from three healthy human donors were cultured in RPMI 1640 (containing 10% fetal calf serum, 100 μM β-mercaptoethanol, 50 μg/ml penicillin, 2 mM glutamine and 50 μg/ml streptomycin) at a density of 1–3 × $10^6$ cells/ml at 37 °C, 5% $CO_2$ in presence of test compounds in 24-well-plates (Sigma, Deisenhofen, FRG). Test compounds were dissolved in dimethylsulfoxide (DMSO, Merck, Darmstadt, FRG) and routinely applied 1 hour before release of TNF-α was stimulated by addition of lipopolysaccharide from *Escherichia coli* serotype 0127:B8 (Sigma, Deisenhofen, FRG) at a final concentration of 2.5 μg/ml. The final concentration of DMSO was adjusted to 0.1 % for each culture sample. After incubation for 20 h at 37 °C and 5% $CO_2$ the TNF-α concentration of all culture supernatants was determined using a commercial TNF-α-ELISA (Boehringer-Mannheim). The percent inhibition of release of TNF-α was calculated relative to cultures treated with DMSO alone. The $IC_{50}$-values were calculated by linear regression analysis.

*Assay for inhibition of IL-2 synthesis by human PBMCs*

PBMCs were isolated and cultured as described above. Test compounds were dissolved in dimethylsulfoxide (DMSO, Merck, Darmstadt, FRG) and 1 μl solution was added to 1 ml culture and incubated for 1 h at 37 °C and 5% $CO_2$. PBMCs were stimulated with 0.1 μg/ml monoclonal antibodies (Klon-Nr. AICD2.M1 Prof. Dr. Meurer; anti CD-28, CLB, Amsterdam) and 0.1 μg/ml staphylococcal-superantigen (*Escherichia coli* serotype 0127:B8,TSST-1, Sigma, Deisenhofen, FRG, ) respectively. After incubation for 20 h at 37 °C and 5% $CO_2$ the IL-2 concentration of all culture supernatants was determined using a commercial TNF-α-ELISA (Boehringer-Mannheim). The percent inhibition of IL-2 synthesis was calculated relative to cultures treated with DMSO alone.

## References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