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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Phthaloyl amino acids as anti-inflammatory and immunomodulatory prototypes

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Abstract A series of phthalimide analogs were synthesized by derivatization of phthalic anhydride, a highly toxic substance, using a "one pot" condensation reaction to α-amino acids. All phthaloyl amino acid derivatives presented anti-oral inflammatory activity, but compounds **2e** and **2g** were found to possess the best activities comparable to thalidomide. Most of the compounds effectively suppressed nitric oxide production in murine cells stimulated with lipopolysaccharide. *N*-phthaloyl amino acids did not exhibit any significant cytotoxicity in vitro when tested against tumor cells as well as a spleen cell culture of BALB/c mice. Compounds **2a**, **2g**, and **2h** were able to inhibit TNF-α and IL-1β production by macrophages. At the same concentration, thalidomide did not exhibit significant inhibitory activity.

Keywords *N*-phthaloyl amino acids · Anti-inflammatory · Immunomodulatory

Introduction

It is well known that the free radicals formed during inflammation play an important role in killing the microorganism and activating leukocytes and macrophages. Overproduction of these radicals is associated with a wide range of pathological conditions (Ariel and Serhan, 2007; Nathan, 2002). Thus, it is important to develop anti-inflammatory and immunomodulatory drugs that could regulate the overproduction of these undesirable species (Moormann *et al.*, 2001).

Phthalimides are well-known plant growth regulators (Butula *et al.*, 1975; Hoffmann and Smith, 1949; Koch, 1971), bacteriostatic agents (Kant and Saksena, 2003; Midtvedt, 1963), and fungicides (Kennedy *et al.*, 1975). Thalidomide, a multi-target drug, is the best-known phthalimide and is a hypnotic/sedative drug with teratogenic effects. Despite these effects, thalidomide has never completely vanished as a therapeutic substance. It was found to have a powerful anti-inflammatory effect due to its ability to inhibit the production of the cytokine tumor necrosis factor alpha (TNF-α), a potent stimulator of inflammation, cellular

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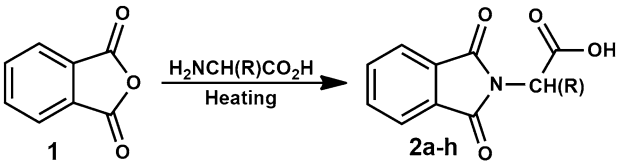
necrosis, and tissue damage in general (Fernández-Martínez *et al.*, 2001). The anticancer activity of thalidomide is based on the inhibition of the growth of new vessels in the process of angiogenesis (Lepper *et al.*, 2004). Thalidomide is being increasingly used in the clinical management of a wide spectrum of immunologically mediated infectious diseases and cancers such as erythema nodosum leprosum, multiple myeloma, renal and intestinal carcinomas, Behçet's, Crohn's, and a number of dermatologic diseases, as well as rheumatoid arthritis and wasting syndrome in AIDS (Franks *et al.*, 2004; Gockel *et al.*, 2004; Kerr and Ship, 2003; Lu *et al.*, 2003; Sayarlioglu *et al.*, 2004; Srinivasan and Lichtenstein, 2004; Zhu *et al.*, 2003). It is also effective for mycobacterial infection in the central nervous system, for example, tuberculous meningitis caused by *Mycobacterium bovis* or *Mycobacterium bacillus* (Tsenova *et al.*, 1999).

Based on the broad spectra of thalidomide properties, the phthalimide pharmacophore has been the target of research with varying intentions. The method by which phthalimide reduces TNF-α production is associated with the induction of the degradation of TNF-α m-RNA (Kim *et al.*, 2004; Lentzsch *et al.*, 2002; Orzeszko *et al.*, 2003; Yogeeswari *et al.*, 2003). *N*-substituted phthalimides are of high interest because they have been reported to possess hypolipidemic activity (Srivastava *et al.*, 2001) as well as anti-inflammatory and immunomodulatory properties (Hashimoto, 2002; Lima *et al.*, 2002; Sena *et al.*, 2003). Phthalimides also have a number of applications in synthetic chemistry (Casimir *et al.*, 2000; Kukolja and Lammert, 1975). In fact, a number of reports have described the synthesis of *N*-phthaloyl amino acids fusing free amino acids with phthalic anhydride (Billman and Harting, 1948; Zeng *et al.*, 2004). To the best of our knowledge, no biological activity tests have been performed for this structural core; therefore, we were interested in carrying out the pharmacological evaluations of these small compounds. This communication reports the results of the in vivo anti-inflammatory tests and immunomodulatory profile. In addition, the compounds were evaluated for their cytotoxicity against human tumor cell lines and a spleen cell culture from BALB/c mice.

Results and discussion

Chemistry

The compounds were synthesized according to described procedures by fusing free amino acids with phthalic anhydride (Fig. 1) (Zeng *et al.*, 2004). It is worth mentioning that only the desired *S*-enantiomers were obtained in all cases, as verified by the addition of the chiral shift reagent Eu(hfc)$_3$ in the ^{1}H-NMR spectra.



Comp.	Entry	R	Comp.	Entry	R
2a	Gly	-H	**2e**	Ile	-$CH_3CH_2(CH_3)CH$
2b	Ala	-CH_3	**2f**	Glu	-$CH_2CH_2CO_2H$
2c	B-Ala	-CH_2CH_2-	**2g**	Phe	-$CH_2C_6H_5$
2d	Val	$CH(CH_3)_2$	**2h**	Trp	CH_2(3-indolyl)

Fig. 1 General synthesis of *N*-phthaloyl amino acid derivatives

Biological activities

Compounds **2a–g** were screened for anti-inflammatory activity in an air pouches model assay with a single dose of 100 mg/kg orally administered to mice and evaluated for their ability to inhibit leukocyte migration from the blood circulation into air pouches (Table 1).

To determine the selectivity and immunomodulatory action, the effect of the derivatives on the proliferation of spleen cells from BALB/c mice (a method that is effective for the evaluation of specific T lymphocyte cytotoxicity) and their effects on NO-induced production in murine spleen cells were tested using thalidomide (Thl) as a reference.

Compounds **2a** and **2b** showed a weak anti-inflammatory profile, whereas **2c**, **2d**, and **2h** presented moderate activity. The derivatives **2e, 2f,** and **2g** (an isoleucine, glutamic acid, and phenylalanine derivative, respectively) were the most potent anti-inflammatory agents. It is

Table 1 Anti-inflammatory activity orally of phthalimides **2a–h** and thalidomide (Thl)

Treated (100 mg/kg)	No. of PMNL/mL ($\times 10^6$)	Inhibition %
Vehicle	5.4 ± 0.1*	–
2a	2.8 ± 0.2*	47.7
2b	3.3 ± 0.2*	38.4
2c	2.5 ± 0.1*	54.2
2d	2.3 ± 0.3*	57.8
2e	1.8 ± 0.2*	67.5
2f	2.1 ± 0.4	61.7
2g	1.9 ± 0.1*	64.2
2h	3.0 ± 0.2*	43.8
Thl	1.5 ± 0.2*	72.1

The data represent the mean ± the standard error of 6 animals

PMNL polymorphonuclear leukocytes

* $P < 0.05$. Significance was determined with one way ANOVA followed by Bonferroni's post hoc test when compared with control group

interesting to note that the amino acid derivative **2f** (a synthetic precursor of Thl) presented a 15 % decrease in anti-inflammatory activity relative to Thl.

Previous work has shown that the inflammatory effects of carrageenan in the air pouch are due to an influx of predominantly neutrophilic leukocytes (mainly PMNL) from the blood circulation, and the reduction in PMNL suggests the inhibition of prostaglandin (PG) production (Jacobs *et al.*, 1981). Therefore, the air pouch model is an attractive method for the direct measurement of PG, TNF-α, and NO, among other inflammatory mediators.

The data shown in Table 2 provide evidence that these phthalimides are efficient at reducing lipopolysaccharide (LPS)-induced nitric oxide production at non-cytotoxic levels and at stimulating the proliferation of spleen cells without affecting cell viability at the dose assayed. These results indicate that these compounds are promising anti-inflammatory agents, especially compounds **2e** and **2g**. Compound **2b** (an alanine derivative) did not provide significant anti-inflammatory activity, but it was able to reduce the production of NO at non-cytotoxic levels.

To better understand the inhibitory effects on NO for the amino acid **2h**, the in vivo biological aspects were investigated using the air pouches model and the NO content of the fluid was measured by the Griess reaction. In the fluid samples from the air pouch, **2h** and Thl (at a dose of 10 mg/kg) significantly decreased the concentration of LPS-induced nitric oxide production compared to the control group (Table 2).

Thereafter, compounds **2e** and **2h** were selected to test the dose-dependent response of the anti-inflammatory activity. When compound **2e** was given at an oral dose of 10 mg/kg, it displayed a 64.7 % inhibition of inflammation, which is comparable to thalidomide (76.3 % inhibition). Compound **2h** (a tryptophyl derivative), which presented poor anti-inflammatory activity orally, was very potent in inhibiting the production of NO, and it did not show cytotoxicity for mammalian cells at a high dose (50 μg/mL) (Fig. 2).

Furthermore, the compounds were evaluated in vitro against a 4-cell-line panel consisting of MDA/MB-435 (human breast), HCT-8 (human colon carcinoma), and SF-295 (human glioblastoma) using a previously described MTT assay (Mosmann, 1983). In this protocol, each cell line is inoculated and preincubated on a microtiter plate. Test agents were then added at a single concentration (25 μg/mL), and the culture was incubated for 48 h. End-point determinations are made with MTT. The results for each test agent were reported as the percent of growth of the treated cells compared to the untreated control cells. Compounds that reduce the growth of any one of the cell lines to 32 % or less are considered cytotoxic (de Moreira *et al.*, 2007). According to these criteria, the derivatives were considered non-cytotoxic (Table 3).

To evaluate the levels of TNF-α and IL-1β, human macrophages were incubated with 50 μmol of compounds **2a–h** over a 24-h time course. These cells were then stimulated with LPS. The purpose of this test was to evaluate the biological capacity of the molecules found to inhibit the synthesis of these extremely important cytokines in inflammatory processes and cancers. The data presented in Fig. 3a show that compounds **2a** and **2h** both inhibit the secretion of TNF-α by macrophages at 50 μmol/mL. In addition, as shown in Fig. 3b, compounds **2a**, **2g,** and **2h** inhibit IL-1β production at the same concentration

Table 2 Inhibitory effects on LPS-induced nitric oxide production and cytotoxic effect on spleen cell BALB/c mice

Treated + LPS[a]	NO-levels (μg/mL) ± SD*		Cytotoxic effects	
	at 25 μg/mL	at 50 μg/mL	Conc. (μg/mL)[b]	% Inhibition[c]
Thl	3.6 ± 0.0*	3.6 ± 0.0*	>100	None
2a	4.2 ± 0.9	3.6 ± 2.3	50	25.60
2b	2.1 ± 0.3*	1.5 ± 0.6*	>100	None
2c	3.6 ± 0.0*	4.4 ± 1.2	50	22.20
2d	6.4 ± 0.0	2.7 ± 0.6*	25	24.69
2e	3.1 ± 0.0*	2.3 ± 0.0*	12.5	26.06
2f	2.9 ± 0.9*	3.4 ± 0.3*	50	08.75
2g	2.3 ± 0.0*	0.7 ± 0.0*	25	21.52
2h	1.3 ± 0.9*	2.1 ± 1.4*	50	27.62

NO-induced production by stimulating with lipopolysaccharide (LPS-1.0 μg/mL) and measured by Griess's method [36]

* $P < 0.05$ compared with control (only LPS stimulus)

[a] NO-induced levels induced by LPS 7.5 ± 1.2 μg/mL

[b] The highest nontoxic concentration on spleen cell of BALB/c mice

[c] % of viable cells

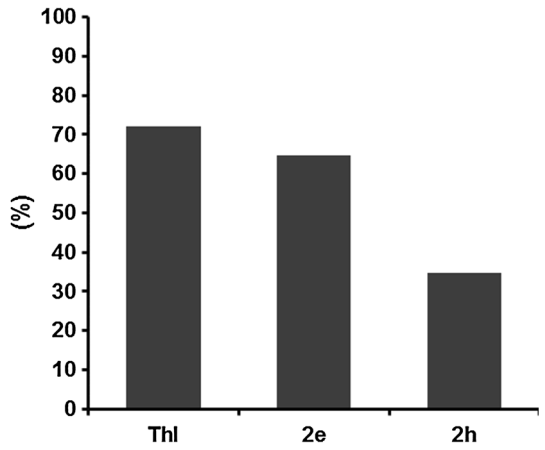


Fig. 2 Anti-inflammatory effects of the compounds **2e, 2h,** and **Thl**. The results are expressed as the percentage of inhibition for $n = 6$ animals. The percent of inhibition was obtained by comparison with a vehicle control group (data not shown) after 6 h (p.o.) of treatment

Table 3 Antiproliferative activity against human cancer cells

Treated	Inhibition of cell proliferation (% ± SD) at 25 μg/mL[a]		
	MDA/MB-435	HCT-8	SF-295
2a	−4.72 ± 0.25	−0.61 ± 2.30	3.98 ± 15.39
2b	5.01 ± 22.38	6.11 ± 2.88	12.21 ± 4.26
2c	−3.71 ± 0.29	18.94 ± 12.53	12.57 ± 7.01
2d	7.82 ± 2.87	4.28 ± 1.87	2.74 ± 5.88
2e	14.95 ± 1.32	15.02 ± 3.67	20.00 ± 6.01
2f	12.92 ± 5.96	6.52 ± 2.88	14.42 ± 7.63
2g	11.72 ± 1.03	−7.23 ± 2.30	13.54 ± 5.38
2h	13.75 ± 2.43	10.44 ± 3.67	24.51 ± 8.39
Dox	94.64 ± 0.93	97.07 ± 4.15	81.36 ± 0.80

[a] Standard deviation is given in parentheses

(50 μmol/mL). Thalidomide did not provide significant inhibitory activity.

Compound **2a** (a phthaloyl-glycine derivative) had presented weak anti-inflammatory activity, but it exhibited an immunomodulatory profile when tested for NO, TNF-α, and IL-1β production. Compound **2g** (a phthaloyl-phenylalanine derivative) showed oral anti-inflammatory activity in the air pouch model, and its immunomodulatory profile showed moderate activity for NO production and strong inhibitory activity for IL-1β production. The phthaloyl-tryptophyl derivative, **2h**, exhibited immunomodulatory activity toward NO, TNF-α, and IL-1β production but poor anti-inflammatory activity with oral administration.

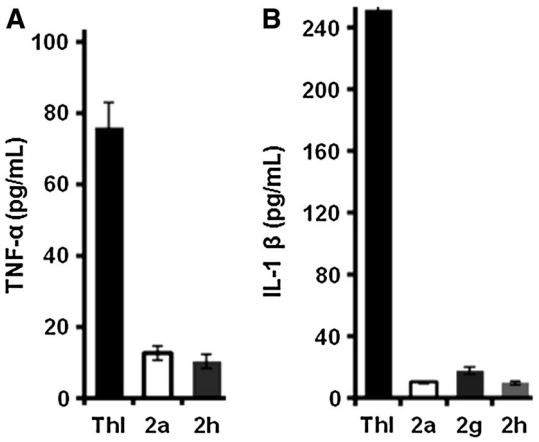


Fig. 3 **a** Effect of the amino acyl phthalimides and Thl in on the production of TNF-α in mouse macrophages (2×10^6 cells/mL) stimulated with LPS (2 μg/mL). TNF-α was measured after 24 h of incubation with compounds (50 μM) by sandwich ELISA (eBioscience kit). Data are the mean ± S.D obtained in triplicate. **b** Effect of phthalimides and Thl on the production of interleukin-1β (IL-1β) in mouse macrophages (2×10^6) stimulated with LPS (2 $\mu g/mL^{-1}$). IL-1β was measured after 24 h of incubation with compounds (50 μM) by sandwich ELISA (eBioscience kit). Data are the mean ± SD (standard deviation) obtained in triplicate. Thl, thalidomide

Conclusion

In summary, *N*-phthaloyl amino acids were readily synthesized and evaluated for their anti-inflammatory activity and cytotoxicity, as well as their NO, TNF-α, and IL-1β production inhibition. These amino acids possess simple structures and could be easily structurally improved. In this series, derivatives from the amino acids, isoleucine and phenylalanine, showed the best oral anti-inflammatory activity in an air pouch model. Moreover, the stimulation of lymphocyte proliferation and the inhibition of NO production suggest that the compounds could be considered as potential immunomodulatory agents. The tryptophyl derivative presented the best immunomodulatory activity of the series. Investigation of immunomodulation and toxicological profiles are subjects for further studies.

Experimental

General

Melting points were determined on an electrothermal capillary melting point apparatus and were uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Varian Unit Plus instrument (300 MHz for protons and 75.5 MHz for carbon). All chemical shifts are reported in ppm relative to TMS as an internal standard. IR spectra were recorded with a Brucker model IFS66 FT-IR spectrophotometer using KBr pellets. All chemicals were purchased from Aldrich, Vetec, or Fluka and were used without additional purification.

Chemistry

All reagents were used as purchased from commercial sources (Sigma-Aldrich, Acros Organics, Vetec, or Fluka). The progress of the reactions was monitored by thin-layer chromatography (TLC) analysis (Merck, silica gel 60 F254 in aluminum foil). The chemical identity was confirmed by NMR and IR spectroscopy and accurate mass (HRMS). IR was performed in KBr pellets. For NMR spectroscopy, we used either a Varian UnityPlus 300 MHz 1H (300 MHz) and 13C (75.5 MHz) or a Bruker AMX-300 MHz 1H (300 MHz) and 13C (75.5 MHz) instrument. DMSO-$_{d6}$ and D_2O were purchased from CIL or Sigma-Aldrich. Chemical shifts are reported in ppm, and multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). NH signals were localized in each spectrum after the addition of a few drops of D_2O. Mass spectrometry experiments were performed on an LC–IT–TOF (Shimadzu). Unless otherwise specified, ESI was carried out in the positive ion mode. Typical conditions were as follows: capillary voltage of 3 kV, cone voltage of 30 V,

and a peak scan between 50 and 1,000 m/z. The compounds were previously synthesized by Zeng et al. (Zeng *et al.*, 2004).

Synthesis

A mixture of 0.5 g (3.38 mmol) of phthalic anhydride **1** and the respective L-amino acid (3.38 mmol) in TEA or 4-DMAP (0.5 mL) and DMF (three drops) was heated by microwave irradiation for 2 min. The crude mixture was then washed with *n*-hexane (30 mL), filtered through a Buchner funnel and recrystallized with 0.1 M $KHSO_4$ aq. or chloroform (40 mL), and dried over anhydrous Na_2SO_4. The compounds **2f** and **2h** were obtained by heating at 180 °C in an oil bath for 10 min.

***(S)-N*-Phthaloylglycine (2a)** yield: 96 %. M.p. (°C):187–191. R*f*:0.5 (8:2-chloroform/methanol). ^{1}H-NMR (300 MHz, ppm):δ 4.15 (s, 2H, CH_2); 7.50–7.70 (m, 4H, Ar); 10.58 (s, 1H, CO_2H). ^{13}C-NMR (75.5 MHz, ppm):δ 39.1 (CH_2); 122.4 (C2 and C5); 132.6 (C1 and C6); 135.8 (C3 and C4); 165.9 (C = O); 174.9 (CO_2H). IR (KBr, cm^{-1}):3212 (νOH); 1775 (νC = O); 1715 (νC = O); 1401 (νC–N–C); 1374 (νC–N–C).

***(S)-N*-Phthaloylalanine (2b)** yield: 70 %. M.p. (°C):135. R*f*:0.46 (8:2-chloroform:methanol). ^{1}H-NMR (300 MHz, ppm):δ 1.70 (d, J = 7.5 Hz, 3H, CH_3); 5.02 (q, J = 7.5 Hz, 1H, CH); 7.70–7.85 (m, 4H, Ar); 10.02 (s, 1H, CO_2H). ^{13}C-NMR (75.5 MHz, ppm):δ 16.9 (CH_3); 51.7 (CHα); 122.4 (C2 and C5); 132.7 (C1 and C6); 135.8 (C3 and C4); 165.6 (C = O); 171.9 (CO_2H). IR (KBr, cm^{-1}):3205 (νOH); 1752 (νC = O); 1740 (νC = O); 1469 (νC–N–C); 1397 (νC–N–C).

***(S)-N*-Phthaloylbetaalanine (2c)** yield: 90 %. M. p. (°C):133–135. R*f*:0.5(8:2-chloroform:methanol). ^{1}H-NMR (300 MHz, ppm):δ 2.50 (t, J = 15.0 Hz, 2H, CH_2); 3.77 (t, J = 15.0 Hz, 2H, CH_2), 7.67–7.51 (m, 4H, Ar), 8.07 (s, 1H, CO_2H). ^{13}C-NMR (75.5 MHz, ppm):δ 29.5 (CH_2); 33.1 (CHα); 122.4 (C2 and C5); 132.6 (C1 and C6); 135.8 (C3 and C4); 165.9 (C = O); 175.9 (CO_2H). IR (KBr, cm^{-1}):3170 (νOH); 1771 (νC = O); 1727 (νC = O); 1466 (νC–N–C); 1411 (νC–N–C).

***(S)-N*-Phthaloylvaline (2d)** yield: 50 %. M.p. (°C):79–81. R*f*:0.60 (8:2-chloroform:methanol). ^{1}H-NMR (300 MHz, ppm):δ 1.9 (d, J = 4.0 Hz, 6H, CH_3); 2.70–2.79 (m, 1H, CH); 4.98 (d, J = 5.0 Hz, 1H, CHα); 8.11 (d, J = 7 Hz, Ar); 8.16 (d, J = 7.0 Hz, Ar); 9.14 (s, 1H, OH). ^{13}C-NMR (75.5 MHz, ppm): δ 18.9 (CH_3); 29.5 (CH); 56.5 (CHα); 122.4 (C2 and C5); 130.7 (C1 and C6); 135.9 (C3 and C4); 165.5 (C = O); 172.9 (CO_2H). IR (KBr, cm^{-1}):3234 (νCO_2H); 1763 (νC = O); 1691 (νC = O); 1469 (νC–N–C); 1401 (νC–N–C).

***(S)-N*-Phthaloylisoleusine (2e)** yield: 74 %. M.p. (°C):110–112. R*f*:0.5 (8:2-chloroform:methanol). ^{1}H-NMR (300 MHz, ppm):δ 0.87 (d, J = 7.2 Hz, 3H); 1.22 (t, J = 6.3 Hz, 3H); 1.50–1.49 (m, 2H); 2.54–2.52 (m, 1H); 4.70 (d, J = 8.4 Hz, 1H); 7.87–7.72 (m, 4H); 9.45 (s, 1H, OH). ^{13}C-NMR (75.5 MHz, ppm):δ 10.6 (CH_3); 16.6 (CH_3); 25.4 (CH_2); 33.9 (CH); 56.5 (CHα); 122.9 (C4 and C9); 131.3 (C5 and C8); 133.8 (C6 and C7); 167.5 (C = O); 170.3 (CO_2H). IR (KBr, cm^{-1}):3250 (νOH); 1763 (νC = O); 1703 (νC = O); 1460 (νC–N–C); 1394 (νC–N–C).

***(S)-N*-Phthaloylglutamine (2f)** yield = 31.9 %. M.p. (°C):204–209. R*f* = 0.58 (8:2-chloroform:methanol). ^{1}H-NMR (300 MHz, ppm):δ 2.48–2.63 (m, 1H, CH_2); 2.11–2.05 (m, 1H, CH_2); 2.96–2.84 (m, 2H, CH_2); 5.13–5.19 (m, 1H, CHα); 7.87–7.90 (m, 4H, Ar); 7.80 (m, 4H, Ar); 11.15 (s, 1H, CO_2H); 11.33 (s, 1H, CO_2H); The 11.15 and 11.33 signals disappear after adding D_2O. ^{13}C-NMR (75.5 MHz, ppm):δ 22.044 (CH_2); 30.995 (CH_2); 49.025 (CHα); 122.967 (C2 and C5); 131.261 (C1 and C6); 134.927 (C3 and C4); 167.217 (C = O); 169.960 (CO_2H); 172.873 (CO_2H). IR (KBr, cm^{-1}): 3201 (νOH); 1775 (νC = O); 1740 (νC = O); 1465 (νC–N–C).

***(S)-N*-Phthaloylphenylalanine (2 g)** yield = 87.4 %. M.p. (°C):167–170. R*f*:0.54 (8:2-chloroform:methanol). ^{1}H-NMR (300 MHz, ppm):δ 3.60 (d, J = 9.0 Hz, 2H, CH_2); 5.258 (t, J = 7.0 Hz, 1H, CHα); 7.15–7.196 (m, 5H, Ar); 7.65–7.68 (m, 2H, Ar); 7.76–7.790 (m, 2H, Ar); 9.01 (s, 1H, OH). ^{13}C-NMR (75.5 MHz, ppm):δ 34.0 (CH_2); 52.9 (CHα); 123.4 (C2′ and C5′); 126.6 (C5 and C9); 128.3 (C6); 128.7 (C8); 130.7 (C7′); 135.0 (C3′ and C4′); 137.3 (C4); 167.2 (C = O); 170.2 (CO_2H). IR (KBr, cm^{-1}):3274 (νCO_2H); 1752 (νC = O); 1704 (νC = O); 1450 (νC–N–C); 1398 (νC–N–C).

***(S)-N*-Phthaloyltryptophan (2 h)** yield = 96 %. M.p. (°C):94–96. R*f* = 0.48 (8:2-chloroform:methanol). ^{1}H-NMR (300 MHz, ppm):δ 3.58 (d, J = 7.00 Hz, 2H, CH_2); 5.15 (t, J = 7.00 Hz, 1H, CHα); 6.89 (t, J = 8.00 Hz, 4H, Ar); 7.03 (m, 4H, Ar); 7.27 (d, J = 9.00 Hz, 4H, Ar); 7.49 (d, J = 9.00 Hz, 4H, Ar); 7.79 (s, 4H, Ar); 10.762 (s, 1H, NH); The 10.762 signal disappear after adding D_2O. ^{13}C-NMR (75.5 MHz, ppm):δ 24.140 (CH_2); 52.680 (CHα); 109.758 (C7); 111.517 (C4); 117.960 (C10); 118.475 (C9); 121.049 (C8); 123.399 (C2′ and C5′); 126.964 (C11); 130.920 (C1′ and C6′); 134.923 (C3′ and C4′); 168.545 (C = O); 170.461 (CO_2H). IR (KBr, cm^{-1}): 3406 (νNH); 3258 (νOH); 1771 (νC = O); 1715 (νC = O); 1464 (νC–N–C).

Biological evaluation

Culture of normal, tumor cells, and cytotoxicity assays

Heparinized human blood (from healthy, nonsmoker donors who had not taken any drugs for at least 15 days

prior to sampling, aged between 18 and 35 years old) was collected, and peripheral blood mononuclear cells (PBMCs) were isolated by a standard method of density gradient centrifugation over Ficoll-Hypaque. All studies were performed in accordance with Brazilian research guidelines (Law 196/96, National Council of Health) and with the Declaration of Helsinki.

Culture of colon (HCT-8)

Glioblastoma (SF-295) and melanoma (MDA/MB-435) cancer lines and PBMC were grown in RPMI 1640 medium supplemented with 20 % fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5 % CO_2. The cytotoxicity of the compounds against human cancer cells was determined by the MTT assay (Mosmann, 1983), which analyzes the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) to a purple formazan product. Briefly, cells were plated in 96-well plates (0.3-0.7 $\times$ 10^5 cells/well) and incubated to allow cell adhesion. Twenty-four hours later, extracts were added to each well (0.04–100 μg/mL). After 72 h of incubation, the supernatant was replaced by fresh medium containing 10 % MTT, the formazan product was dissolved in DMSO, and the absorbance was measured at 595 nm. Quantification of cell proliferation was determined spectrophotometrically using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter). Control groups (negative and positive) received the same amount of DMSO (0.1 %). Doxorubicin (Dox) (0.02–8.6 μM) was used as a positive control.

Preparation of splenocytes

Mouse splenocytes were obtained by Pereira et al. (Hernandes *et al.*, 2010). After killing the animal with CO_2 gas, the spleen of each mouse was removed aseptically and placed in a Falcon tube containing RPMI 1640 with fetal calf serum (complete medium). In a vertical flow, each spleen was transferred to a Petri dish where they were soaked. The cell suspensions obtained were transferred to Falcon tubes containing approximately 10 mL of incomplete medium by spleen. Spleen homogenates were overlaid onto a Ficoll-Paque™ PLUS layer with the density adjusted to 1.076 g/mL and centrifuged at 1,000×*g* at room temperature for 25 min. The interface cell layer containing immune cells was recovered by a Pasteur pipette, washed twice in PBS, and centrifuged two times at 500×*g* for 10 min. Cells were counted in a Neubauer chamber, and cell viability was determined by the trypan blue exclusion method. Cells were used only when viability was greater than 98 %.

In vitro cytotoxicity assays

The cytotoxicity of compounds **2a–h** was determined using BALB/c mice splenocytes (6 $\times$ 10^5 cells/well) cultured in 96-well plates in RPMI 1640 media (Sigma Chemical Co., St. Louis, MO) supplemented with 10 % fetal bovine serum (FCS; Cultilab, Campinas, SP, Brazil) and 50 μg/mL of gentamycin (Novafarma, Anápolis, GO, Brazil). Each compound was evaluated at six concentrations (100, 50, 25, 10, 5, and 1 μg/mL) in triplicate in two independent assays. Cultures were incubated in the presence of [^{3}H]-thymidine (Amersham Biosciences) (1 μCi/well) for 24 h at 37 °C and 5 % CO_2. After this period, the content of the plate was harvested to determine the [^{3}H]-thymidine ([^{3}H]TdR) incorporation using a beta-radiation counter (β-matrix 9600, Packard). The toxicity of compounds **2a–h** was determined by comparing the percentage of [^{3}H]-thymidine incorporation (as an indicator of cell viability) of lectin-treated wells compared to untreated wells. Saponine (0.05 %), concanavalin A (Con A), and phytohemagglutinin (PHA) were used as positive controls. Non-cytotoxic concentrations were defined as those causing a reduction in [^{3}H]-thymidine incorporation below 30 % in relation to untreated controls.

Carrageenan-induced air pouch

The anti-inflammatory activity of the compounds was tested by the formation of air pouches on the dorsal cervical region of mice (25–30 g) by subcutaneous injection of 2.5 mL of sterile air on day 0 followed by a second injection of 2.5 mL of sterile air 3 days later. On the 6th day, the mice received vehicle or the test compounds orally. One hour after drug administration, inflammation was induced by injecting 1 mL of carrageenan suspension (1 % in saline solution) into the air pouch. After 6 h, the mice were killed and the lumen of the air pouch was lavaged by intrapouch injection of 3 mL of PBS containing 50 ng/mL heparin and subsequent aspiration of the fluid. Total numbers of PMNL cell infiltration were determined with a Neubauer hemocytometer.

In vitro nitrite analysis

The mice spleen cells were used to evaluate the concentration of nitrite after treatment with LPS and the compounds **2a–h** (at 25 and 50 μg/mL), and after 72 h of incubation, the media were carefully collected for measurement by the colorimetric Griess method (Ding *et al.*, 1988). The NO concentration was estimated by a standard curve of sodium nitrite (3.12-50 μmol/mL).

TNF-α and IL-1β levels

Mice peritoneal macrophages were placed into 96-well plates at a cell density of 2×10^6 cells/mL and incubated for 2 h at 37 °C and 5 % CO_2. Cells at a concentration of 2×10^6 were suspended in RPMI 1640 with 5 % FBS, 100 UI/mL of penicillin, 100 μg/mL of streptomycin, and 50 mM 2-mercaptoethanol. One hundred microliters of the suspension and 100 μL of the samples were incubated with 2 μg/mL LPS (positive control) or with the test compounds in different concentrations. After 24 h, the supernatants were removed and kept at −80 °C until the evaluation of cytokine levels (TNF-α and IL-1β). The doses of cytokines in the exudates were assayed by sandwich ELISA using monoclonal antibodies specific to the detection of the cytokine.

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Conflict of interest The authors state no conflict of interest.

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