Article

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Characterization of Novel Aminobenzylcantharidinimides and Related Imides by Proton NMR Spectra and Their Effects on NO Induction

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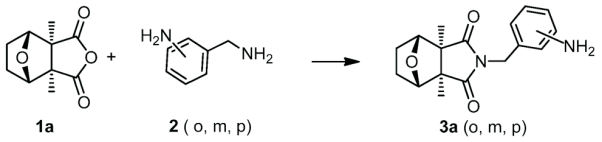
Various acidic anhydrides including cantharidin were converted into corresponding aminobenzylcantharidinimide **3a** and analogous imides **3b~k** (at the *ortho*, *meta,* and *para* positions) with 35%~87% yields by reacting with aminobenzylamines and triethylamine. The two methyl side chains of cantharidinimides **3ao**, **3am**, and **3ap,** and related imides had more than two chiral centers; the lone pair of electrons of nitrogen displayed a different chemical shift and coupling constant in H-NMR spectra when the amino group of benzylamine was in the *ortho* position. These cantharidinimides had parent aniline, pyridine, and naphthalene plane structures, and the primary amine nucleophilicity and basicity might reflect the inductive electron's negative effect on chemical shifts. We prepared cantharidinimides by heating the reactants cantharidin **1a**, aliphatic and aromatic acid anhydrides, primary benzylic amines, and aniline derivatives to ca. 200 °C with 3 mL of dry toluene, and 1~2 mL of triethylamine in high-pressure sealed tubes (Buchi glasuster 0032) to produce cantharidinimides and their analogues in good yields. The *para*-aminobenzylic imides showed greater inhibition of nitric oxide (NO) synthesis by NO synthase (NOS) than did *ortho*- and *meta*-aminobenzylic imides. Compound **3fp**, *para*-aminobenzylic norbonane-imide, had the most potent effect on inducible NOS among the tested compounds and showed 35% inhibition.

Keywords: Cantharidin; Cantharidinimide; Aminobenzylamine; iNOS.

INTRODUCTION

Cantharidin is found in *Mylabris caraganae* and various other insects and shows extremely high vesicant potency and toxic properties.[1-3] In clinical studies, it was shown to possess antitumor and antihepatoma properties. However, it cannot be applied in the clinic due to its high potency and toxic properties.[4-8] It is only used as a standard in research in veterinary medicine due to its irritant and vesicating effects. In a search for less-toxic analogues of cantharidin or cantharidinimide derivatives, a slightly modified structure was synthesized in an analogous manner.[9] Cantharidin (**1a**) was subjected to an oxygen-nitrogen exchange reaction to convert it into cantharidinimide (**3a**) by reaction with primary amines at 200 °C (Scheme 1). Because acidic anhydrides are recognized as a class of analogous compounds, some of them, such as phthalic anhydride (**1b**), 4-methylphthalic anhydride (**1c**), 3-methylphthalic anhydride (**1d**), 1,8-naphthalenedicarboxylic anhydride (**1e**), methylnorbonene-2,3-dicarboxylic acid anhydride (**1f**), citraconic anhydride (**1g**), himic anhydride (**1h**), and 2,3-pyridinecarboxylic anhydride (**1i**), can be used to prepare corresponding imides under similar conditions. We also used diphenic anhydride (**1j**), 3-nitrophthalic anhydride (**1k**), and 2,3-dimethylmaleic anhydride (**1l**) to investigate the results of various reactions (Scheme 2). Previous work showed that yields depended on the basicity of compound **2** and perhaps reflected the inductive effects of electron-withdrawing and -donating groups on the aminoben-

Scheme 1 Synthesis of **3a**



1a **2** (o, m, p) **3a** (o, m, p)

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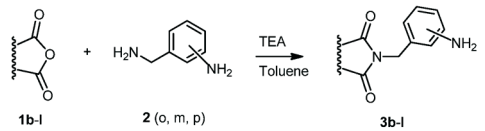
Scheme 2 Synthesis of **3b-l**



b: Phthalic anhydride
c: 4-Methyphthalic anhydride
d: 3-Methylphthalic anhydride
e: 1,8-Naphthalenedicarboxylic acid anhydride
f: Norbonan-2,3-dicarboxyl acid anhydride
g: Citraconic anhydride
h: Himic anhydride
i: 2,3-Pyridindicarboxylc acid anhydride
J: Diphenic anhydride
k: 3-Nitrophthalic anhydride
l: 2,3-Dimethylmaleic anhydride

zylamine ring, which might have influenced the charge density at the benzene ring and thus affected the reactivity. The temperature was also crucial in these reactions.

Nitric oxide (NO) has important roles in human regulatory systems such as vasodilation and platelet aggregation in the cytostatic and cytotoxic actions of macrophages and neutrophils.[10] NO-induced vasodilation is mediated by cyclic guanosine monophosphate (c-GMP).[11] In addition, it was reported that NO increases the activity of soluble guanylate cyclase in vascular smooth muscle, which results in vasodilation. Our synthetic compounds exhibited a certain degree of cytotoxic activity against HL-60 and Hep3B hepatocellular carcinomas.[12] Herein, we report our systematic studies of various acidic anhydrides to produce imides under a base condition (Scheme 2) and the effects of cytotoxicity and as inducible NO synthase (iNOS) inhibitors for designing new drugs as future therapeutic sources.

RESULTS

We treated cantharidin and various acidic anhydrides **1a**~**k** in 3 mL of toluene with 1~2 mL of triethylamine, *ortho*-, *meta*-, and *para*-aminobenzylamines, and heating to ca. 200 °C to yield the corresponding imides **3a**~**k** and **4a**~**k** (Table 1). We successfully converted **1a**~**k** into **3a**~**k,** respectively, in yields of 35%~87%. The **4a**~**k** imide compounds failed to yield anything, and the high regiospecific reaction could be explained by the pK_a values of the two amino groups of aminobenzylamine. The results obtained only with **3a-k** strongly confirmed the influence of the amine basicity. It seemed that aliphatic amines were predominant over aromatic amines, and the preparative techniques and steric hindrance were also factors influencing yields (Table 1). We found that *para*-aminobenzylamines were converted into the products, **3ap**~**kp**, in yields of 16%~87%, while *ortho*-aminobenzylamines gave products **3ao**~**3ko** in yields of 7%~85%, and *meta*-aminobenzylamines gave products **3am**~**3dm** in yields of 65%~75%.

Table 1. Conversion of acidic anhydride into the corresponding imides by TEA

Acetic anhydride	mmol			product (% yield)[b]		
1	**2**[a] **o**	**m**	**p**	**3 o**	**m**	**p**
1a	1.5	1.5	1.5	46	71	85
1b	1.2	1.2	1.2	66	75	85
1c	1.1	1.1	1.1	70	65	73
1d	1.1	1.1	1.1	70	75	79
1e	1.1	-	1.1	35	-	43
1f	1.1	-	1.1	40	-	45
1g	1.2	-	1.2	85	-	87
1h	1.2	-	1.2	76	-	78
1i	1.1	-	1.1	37	-	41
1j	1.1	-	1.1	15	-	20
1k	1.1	-	1.1	7	-	16
1l	1.1	-	1.1	30	-	-

[a] **2 o**, **m**, **p** = Aminobenzylamine, *ortho*, *meta*, *para*.; [b] After purification by chromatography on silica gel and then recrystillized with methanol.; Conditions: TEA/toluene (1/3, mL), 200 °C, 2 h.

Table 2. Tests of Nitric oxide synthase

LPS[a]+	Nitrite (mM)	% of Inhibition
None	33.1	
0	-0.1	100.4
Th[b]+-	24.7	25.6
Th+	24.2	26.9
Th-	23.8	28.2
3bo	15.5	53.1
3co	0.5	98.4
3eo	11.4	65.6
3fo	8.1	75.5
3lo	9.0	72.8
3bm	2.7	91.9
3em	18.8	43.3
3bp	6.2	81.4
3dp	6.4	80.7
3ep	18.3	44.6
3fp	21.4	35.4

[a] LPS: lipopolysaccharide; [b] Th: Thalidomide.

Inhibitory percentages of NO synthesis and nitrite concentrations after treatment with synthetic compounds **3bo**~**3lo** are shown in Table 2. All tested compounds showed NO inhibition, and the most potent compound was **3fp**, a *para*-aminobenzylic norbonane-imide.

DISCUSSION

In studies on cantharidin **1a**, it was found that the *J*

value of CHO was in the triplet at δ 4.72, and the coupling constant was 2.4 Hz. Signals of the two methyls had equal intensities to that at δ 1.23, but were nonetheless very sharp. The identity of 2-aminobenzylcantharidinimide (**3ao**) was confirmed by NMR as shown in Fig. 1. We found that when the amino group was in the *ortho* position of the benzylamine of cantharidinimide, δ and *J* values were more complicated, and when compared to the properties and spectral data of **3am** and **3ap**, we noted one complication in the NMR spectrum of compound **3ao**, which showed two methyl assignments that were not equivalent. They appeared at δ 1.13 and 1.22, with each showing one singlet. Signals of the two benzylic protons displayed different equivalent hydrogens in this structure of the type, *N*-CH_2-C, which appeared at δ 4.79 and showed two doublet peaks, with *J* values of 16.1 and 16.2 Hz. The proton chemical shift of CHO was at δ 4.53 and displayed two doublets; its coupling constant was 3.6 Hz. The fact that there were three different signals, each resulting from three different kinds of equivalent hydrogens, suggested a high degree of asymmetry in the structure, as if one methyl of cantharidinimide was on the same side with the *ortho* amine which exhibited δ 1.22. By a lucky chance, clear and sharp spectra of the two species were obtained at about 25 °C (Figs. 1, 2), i.e., at a normal temperature of the spectrometer. A part of the crude crystal product mixture of **3ao** prior to chromatography gave these spectra. Despite many efforts, it was not possible to reproduce a spectrum that indicated slow isomerization between the two species in the absence of an unknown catalyst or solvent. In a discussion of Fig. 1, isomerization was accelerated by either the solvent, reaction time, or temperature in the NMR determination.

Compared to Fig. 2, aromatic protons with a small shift differed between species, namely in signals for the two methyl groups and OC-H of the ring. It was proposed that the two species of **3ao** are syn-anti isomers **A** (minor) and **B** (major) with preferred conformations as in the formula shown in Fig. 3. The conformation of **A** was favored by the hydrogen bond and steric repulsion between the two methyl groups and benzyl (phenyl), while conformation of **B** possibly exhibited the least electrostatic repulsion between the lone nitrogen pair and oxygen of bridge atoms with their partial negative charge. The difference in **A** relative to **B** was observed to be the *ortho* proton of benzyl (phenyl), and this shift occurred for several possible reasons: the hydrogen bond of the amino group might have enhanced the electro capacity of the *ortho* phenyl hydrogen, thus shifting the signal downfield from the usual range. For **B** this signal might have been shifted upfield by an electric field effect arising from the lone nitrogen pair and by the diamagnetic anisotropy of the C=O bond.

In the mass spectrum of **3ao**, the molecular peak at 300 did not show the cyclization form between the carbonyl and amine. This might possibly have occurred due to hydrogen bonding and have become a more-stable six-ring-like conformation or might simply have been due to

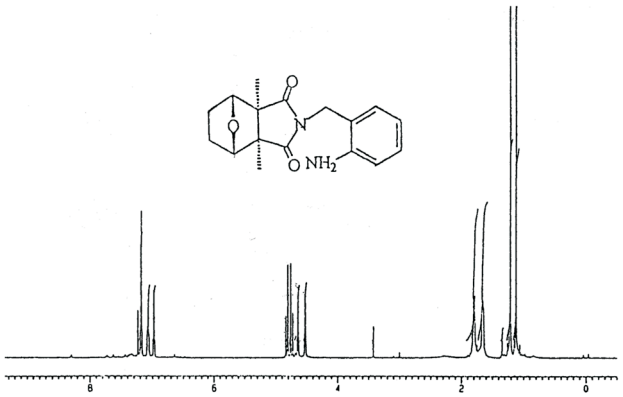


Fig. 1. The proton NMR ($CDCl_3$) of compound **3ao** (type **A**).

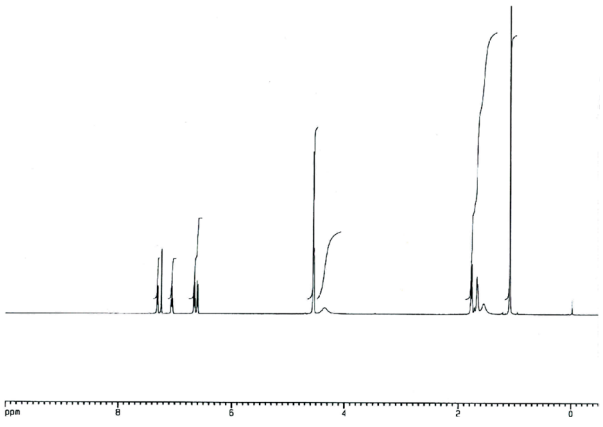


Fig. 2. The proton NMR ($CDCl_3$) of compound **3ao** (type **B**).

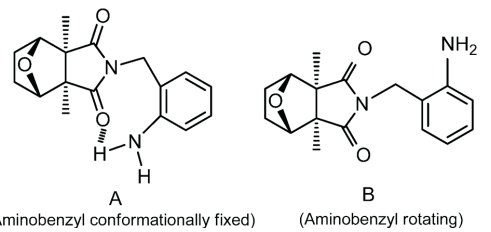


Fig. 3. Structure of aminobenzylcantharidinimide conformers A and B.

nitrogen's polar effects. We did not find it in compounds **3am** or **3ap**, as their methyl and benzylic hydrogens seemed to be equivalent, with a sharp methyl signal at δ 1.12 and benzylic H at δ 4.54. We found that the benzylic protons of compounds **3bo**, **3co**, **3eo**, and **3io** had an aromatic phthalic imide and an aliphatic maleic imide and revealed a planar structure; their benzylic protons showed sharp singlet peaks at δ 4.92 and 4.73, and methyl groups had singlet peaks with two different values at δ 1.97 and 2.13. Compounds **3bm**, **3cm**, **3dm**, **3em**, and **3im** had respective sharp singlet peaks at δ 4.73, 4.83, 4.83, 5.27, and 4.79, and their *para* derivatives, **3bp**, **3dp**, **3ep**, and **3hp**, also had respective sharp singlet peaks at δ 4.71, 4.68, 5.25, and 4.52 that produced no special changes. The benzylic protons of **3ho** showed some coupling signals compared to those of compound **3ao**, and we found its benzylic protons were at δ 6.16 and displayed a double doublet signal. This might be explained by both compounds having a chiral center in their structures and different steric conformations which might be factors influencing the NMR chemical shift of the hydrogens, principally their molecular environments. We also found that the two methyls of *o*-aminobenzyl-2,3-dimethylmaleic imide were not equivalent and exhibited two sharp singlets at δ 1.97 and 2.13, but the chemical shift of its benzylic proton was within the expected position at about δ 4.73 as a singlet peak.

These compounds exhibited less cytotoxicity than other anticancer drugs currently being used; thus their derivatives could be candidates for testing as NO-inhibitory materials. Our tested compounds **3bo**~**3lo** had structures similar to that present in thalidomide, which has two imide structures, phthalimide and glutarimide. It inhibited NO synthesis by 25%~28% (Table 2). Hence, we used it as a standard to test the inhibition of NO formation. Based on the pharmacological results, it was also possible to make a number of correlations between the structure and inhibition of NO synthesis by NO synthase with the 11 imide compounds. It was found that the inhibition of NO formation by NOS of *N*-(aminobenzyl)phthalimide derivatives decreased in the following order: **3bo** > **3bp** > **3bm**. It was shown that the *ortho*-amino group of benzylphthalimide might display some pharmacological effect better than the others. It demonstrated that the amino group in the *ortho* position might create more-stable and comformationally fixed isomers that were predominant over the others. Comparing the bioactivity of *N*-(2-aminobenzyl)imides (**3bo**, **3co**, **3eo**, **3fo**, and **3lo**) revealed that naphthalene ring has the less slightly ability to inhibit NOS activity as unsubstituted benzene ring. It was revealed that the inhibition of NO synthesis by NOS of *N*-(aminobenzyl)-1,8-naphthalimide derivatives decreased in the following sequence: **3em** ≈ **3ep** > **3eo**. It was shown that the *meta*/*para*-amino group was better than the *ortho*-amino group in the high lipophilicity of the naphthalene moiety which might have more effect for NO inhibition. Comparing the bioactivity of *N*-(2/4-aminobenzyl)norborane-imides revealed that the *para*-amino group was better than the *ortho*-amino group on the norborane ring which might have same effect as cantharidinimide. Among the tested compounds, we found that *N*-(4-aminobenzyl)norboraneimide (**3fp**) showed the most potent effect on iNOS at 35% inhibition. We supposed that the *para*-amino group and the spherical norborane moiety might be linked at a position opposite of the receptor.

EXPERIMENTAL

General: Melting points were determined with a melting point microscope (Yanaco apparatus). Silica gel (0.063~0.200 mm, 70~230 mesh) supplied by Merck was used for column chromatography. Infrared (IR) spectra of KBr discs were recorded on Bio-Ras FT-IR FTS 165 and Nicolet 510 PFT-IR spectrophotometers. Mass spectra were obtained on Joel JMSHX 110 FABMS and Joel-HX 110 high-resolution spectrometers. ^{1}H nuclear magnetic resonance (NMR) spectra ($CDCl_3$ unless otherwise stated) were recorded at 500 MHz on a Bruker Advance DRX. Chemical shifts are shown as δ values (ppm) with tetramethylsilane (TMS) as an internal reference.

General procedure for the synthesis of cantharidinimides and imide analogues: Cantharidinimides and imide analogues were prepared from cantharidin and aminobenzylamines in the presence of triethylamine and toluene in high-pressure tubes with stirring and heating to ca. 200 °C. After being stirred for 2 h, the mixture was evaporated, and the residue was purified by column chromatography on silica gel and then recrystallized from methanol.

Physical properties of 3ao, 3am, 3ap, 3bo, 3co, 3eo, and 3lo: *N*-(2-Aminobenzyl)cantharidinimide (3ao): Cantharidin (204 mg, 1.1 mmol), amine (140 mg, 1.2 mmol), TEA (1 mL), toluene (3 mL); mp 133~136 °C (MeOH); IR (KBr) 1726 (amide), 3569 (NH_2) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.13 (3H, s, CH_3), 1.22 (3H, s, CH_3), 1.66-1.82 (4H, m, CH_2 x2), 4.53 (1H, d, J = 3.6 Hz, OCH), 4.65 (1H, d, J = 3.6 Hz, OCH), 4.79 (2H, dd, J = 16.1 , J = 16.2 Hz, benzylic H), 6.98 (1H, d, J = 7.5 Hz, H-3), 7.09 (1H, dd, J = 3.9, 7.5 Hz, H-5), 7.18 (2H, d, J = 3.9 Hz, H-2 overlapping

with H-4); MS *m/z* (rel. int. %) 300 (M^+, 5), 281 (10), 57 (100); HRMS (EI, 80 eV) calcd. for $C_{17}H_{20}O_3N_2$ 300.1474, found 300.1480. ***N*-(3-Aminobenzyl)cantharidinimide (3am)**: Cantharidin (199 mg, 1.1 mmol), amine (135 mg, 1.1 mmol), TEA (1 mL), toluene (3 mL); mp 175~179 °C (MeOH); IR (KBr) 1692 (amide), 3355, 3417 (NH_2) cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.61 (1H, s, H-2), 6.54 (1H, dd, *J* = 1.9, 7.8 Hz, H-4), 6.67 (1H, d, *J* = 7.8 Hz, H-6), 7.05 (1H, d, *J* = 7.8 Hz, H-5); MS *m/z* (rel. int. %) 300 (M^+, 100), 231 (10),106 (65); HRMS (EI-80 eV) calcd. for $C_{17}H_{20}O_3N_2$ 300.1474, found 300.1479. ***N*-(4-Aminobenzyl)cantharidinimide (3ap)**: Cantharidin (206 mg, 1.2 mmol), amine (256 mg, 2.0 mmol), TEA (1 mL), toluene (3 mL); mp 202~205 °C (MeOH); IR (KBr) 1693 (amide), 3367, 3444 (NH_2) ; ^1H NMR ($CDCl_3$) δ 1.11 (6H, s, CH_3), 1.64-1.83 (4H, m, CH_2 x 2), 4.53 (2H, d, *J* = 14.1 Hz, CH_2N), 4.56 (2H, s, OCH), 6.57 (2H, d, *J* = 8.2 Hz, phenyl 3,5-H), 7.09 (2H, d, *J* = 8.1 Hz, phenyl 2,6-H), MS *m/z* (rel. int. %) 300 (M^+, 30), 106 (100). HRMS (EI-80 eV) calcd. for $C_{17}H_{20}O_3N_2$ 300.1474, found 300.1476. ***N*-(2-Aminobenzyl)-phthalimide (3bo)**: Phthalic anhydride (150 mg, 1.0 mmol), amine (130 mg, 1.1 mmol), TEA (1 mL), toluene (3 mL); mp 310~313 °C (MeOH); ^1H NMR ($CDCl_3$) δ 4.73 (1H, s, benzylic H), 7.14 (1H, d, *J* = 7.4 Hz, H-3'), 7.22 (1H, dd, *J* = 9.6, 7.4 Hz, H-5'), 7.31 (1H, t, *J* = 7.5 Hz, H-4'), 7.45 (1H, d, *J* = 7.7 Hz, H-6'), 7.65 (1H, d, *J* = 7.4 Hz, H-4), 7.67 (1H, d, *J* = 7.4 Hz, H-5), 7.88 (1H, d, *J* = 7.2 Hz, H-3), 8.03 (1H, d, *J* = 7.4 Hz, H-6); Cl-MS *m/z* (rel. int. %) 252 (M^+, 17), 104 (22), 76 (100). ***N*-(2-Aminobenzyl)-4-methylphthalimide (3co)**: mp 133~135 °C (MeOH); IR (KBr) 1714 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.48 (1H, s, CH_3), 7.13 (2H, dd, *J* = 7.3, 7.4, phenyl H-3', H-4'), 7.26-7.30 (2H, m, phenyl H-5'), 7.44 (2H, m, benzene H-5', phenyl H-6'), 7.74 (1H, s, benzene H-3), 7.89 (1H, t, *J* = 7.7 Hz, benzene H-5); MS *m/z* (rel. int. %) 266 (M^+, 17), 104 (22), 76 (100). ***N*-(2-Aminobenzyl)-1,8-naphthalimide (3eo)**: mp 173~175 °C (MeOH); IR (KBr) 1713 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.28 (2H, s, benzylic H),7.16 (2H, dd, *J* = 6.5 Hz, benzylic 3,4-H), 7.24 (1H, m, benzylic H-5), 7.36 (1H, s, benzylic H-6), 7.66 (1H, t, *J* = 3.8 Hz), 7.68 (1H, d, *J* = 7.8 Hz), 8.03 (1H, t, *J* = 3.8 Hz), 8.10 (1H, d, *J* = 3.1 Hz), 8.45 (1H, d, *J* = 8.1Hz), 8.83 (1H, d, *J* = 7.6 Hz). MS *m/z* (rel. int. %) 302 (M^+, 17), 104 (22), 76 (100). ***N*-(2-Aminobenzyl)-2,3-dimethylmaleiimide (3lo)**: mp 175~178 °C (MeOH); IR (KBr) 1713 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.97 (3H, s, 3-CH_3), 2.13 (3H, s, 2-CH_3), 4.73 (2H, s, benzylic CH_2), 7.13 (1H, dd, *J* = 7.5, 7.5 Hz, H-3'), 7.26 (1H, d, *J* = 7.5 Hz, H-4'), 7.42 (1H, d, *J* = 7.1 Hz, h-5'), 7.71 (1H, d, *J* = 3.3 Hz, H-6'); MS *m/z* (rel. int. %) 220 (M^+, 17), 104 (22), 76 (100). **Nitrite quantitation:** NO_2-accumulation was used as an indicator of NO production in the medium as previously described.[13] Raw 264.7 cells at 4~5 mL were maintained in 96-well, 24-h culture plates; 20 μg/ml of compounds was added and stimulated with lipopolysaccharide (LPS) (300 ng/mL). After 18 h, isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 5% phosphoric acid) and incubated at room temperature for 10 min using Na_2NO_2 to generate a standard curve. Nitrite production was measured with an enzyme-linked immunosorbent assay (ELISA) reader at 530 nm.

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