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Furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole derivatives as potential inhibitors of inflammatory factors in sepsis

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ABSTRACT

Synthesis and anti-inflammatory effects of certain furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole derivatives **12–18** were studied. These compounds were synthesized from naphtho[1,2-*b*]furan-4,5-dione (**10**) which in turn was prepared from the known 2-hydoxy-1,4-naphthoquinone (**7**) in a one pot reaction. Furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole (**12**) was inactive (IC_{50} value of >30 μM) while its 5-phenyl derivative **13**, with an IC_{50} value of 16.3 and 11.4 μM against lysozyme and β-glucuronidase release, respectively, was comparable to the positive trifluoperazine. The same potency was observed for 5-furan derivative **16** with an IC_{50} value of 19.5 and 11.3 μM against lysozyme and β-glucuronidase release, respectively. An electron-withdrawing NO_2 substituted on 5-phenyl or 5-furanyl group led to the devoid of activity as in the cases of **14** and **17**. Among them, compound **15** exhibited significant inhibitory effects, with an IC_{50} value of 7.4 and 5.0 μM against lysozyme and β-glucuronidase release, respectively.
For the LPS-induced NO production, the phenyl derivatives **12–15** were inactive while the nitrofuran counterparts **17** and **18** suppress LPS-induced NO production significantly, with an IC_{50} value of 1.5 and 1.3 μM, respectively, which are more active than that of the positive 1400 W. Compounds **16–18** were capable of inhibiting LPS-induced iNOS protein expression at a dose-dependent manner in which compound **18**, with an IC_{50} of 0.52 μM in the inhibition of iNOS expression, is approximately fivefold more potent than that of the positive 1400 W. In the CLP rat animal model, compound **18** was found to be more active than the positive hydrocortisone in the inhibition of the iNOS mRNA expression in rat lung tissue. The sepsis-induced PGE2 production in rat serum decreased 150% by the pretreatment of **18** in a dose of 10 mg/kg.

1. Introduction

Mast cells, neutrophils, and macrophages are important inflammatory cells that have been implicated in the pathogenesis of acute and chronic inflammatory diseases. Activated neutrophils release lysozyme and β-glucuronidase which are capable of proteolytic disruption of healthy tissue in a number of disease states such as pulmonary emphysema, rheumatoid arthritis, arteriosclerosis, and glomerulonephritis.[1,2] Macrophages are the main pro-inflammatory cells responsible for invading pathogens by releasing many pro-inflammatory molecules, including the free radical nitric oxide (NO) which is produced endogenously by the nitric oxide synthase (NOS). Three isoforms of NOS exist, constitutively expressed neuronal NOS (nNOS) and endothelial NOS (eNOS), and inducible isoform (iNOS).[3] Activated macrophages transcriptionally express inducible nitric oxide synthase (iNOS), which is responsible for the prolonged and profound production of NO.[4–6] The aberrant release of NO can lead to amplification of inflammation as well as tissue injury.[7] Therefore, inhibition of neutrophils and/or macrophages activation and the following release of inflammatory mediators provide a promising strategy for the development of potential anti-inflammatory agents.

Many efforts have been devoted to the discovery of novel anti-inflammatory agents for the past few years.[8–13] We have also synthesized certain 9-anilinoacridine, 9-phenoxyacridine, 4-phenoxyfuro[2,3-*b*]quinoline, and quinolin-2(1*H*)-one derivatives and evaluated for their anti-inflammatory activities (Fig. 1).[14–19] Among them, 4-anilinoacridine derivatives[14,15] such as compound **1**, 4-anilinofuro[2,3-*b*]quinoline derivatives[16] such as compound **2**, and 4-phenoxyquinoline derivatives[18] such as compound **3** have shown potent inhibitory activities for the secretion of lysozyme

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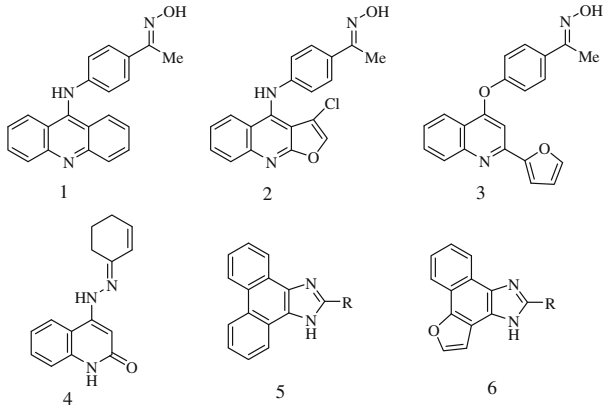


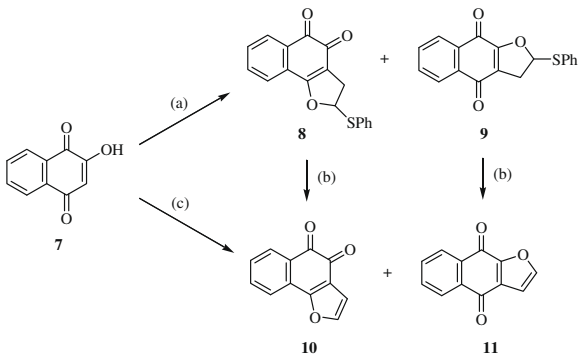
Figure 1. Structure of compounds **1–6**.

and β-glucuronidase from neutrophils, with IC_{50} values in a range of 4.4 and 11.6 μM. Quinolin-2(1*H*)-one derivatives[19] such as compound **4** significantly suppressed LPS-induced NO production and iNOS gene expression in macrophages.

Prostaglandin E2 (PGE2) is widely recognized as a key mediator in fever, pain, and inflammatory response. Extensive studies had been devoted recently to explore selective PGE2 modulators which act through downstream microsomal PGE synthase-1 (mPGES-1) inhibition. A number of substituted imidazo[4,5-*f*]phenanthrene derivatives (**5**)[12] have been identified as potent and selective mPGES-1 inhibitors. To continue our study in the development of potential anti-inflammatory drug candidates, the present report describes the preparation and evaluation of certain furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole derivatives (**6**) which can be considered as isosteric isomers of **5**.

2. Chemistry

Preparation of naphtho[1,2-*b*]furan-4,5-dione (**10**) had been reported by the two-step reaction from 2-hydroxy-1,4-naphthoquinone (**7**).[20] Treatment of **7** with phenylvinylsulfide in the presence of ceric ammonium nitrate (CAN) gave angular dihydrofuronaphthoquinone **8** and its linear isomer **9**. Oxidative elimination of **8** with *m*CPBA afforded **10** as described in Scheme 1. Instead of following these known procedures, we adapted a more facile one pot reaction which was similar to our previous preparation of furo[3,2-c]quinoline.[21] Thus, **7** was reacted with chloroacetaldehyde to give a mixture of angular naphtho[1,2-*b*]furan-4,5-dione (**10**) and linear naphtho[2,3-*b*]furan-4,9-dione (**11**) in a yield of 40% and 10%, respectively.



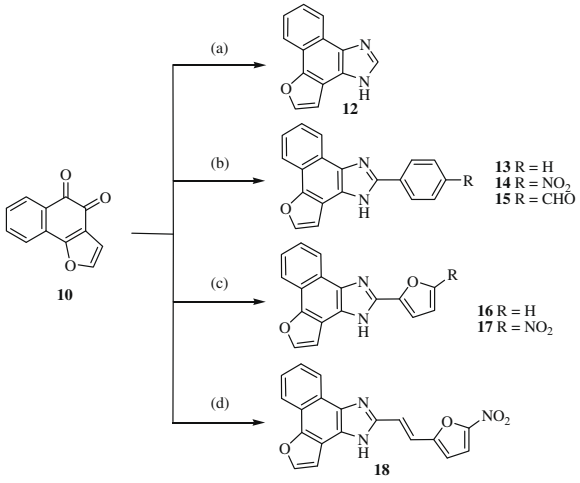
Scheme 1. Reagents and conditions: (a) phenylvinylsulfide, CAN, CH_3CN, 0 °C, 6 h; (b) *m*CPBA, CH_2Cl_2, rt, 12 h (procedures of Lee et al.;[20] (c) 40% $ClCH_2CHO_{(aq)}$, KI, KOH, reflux, 4 h.

Treatment of **10** with ammonium acetate and paraformaldehyde afforded furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole (**12**) in 61% yield as depicted in Scheme 2. Structure of **12** was confirmed by NMR spectra and elemental analysis. Accordingly, 5-aryl substituted derivatives **13–18** were prepared from **10** and their respective aryl aldehyde derivatives.

3. Results and discussion

3.1. Neutrophil degranulation

Activation of neutrophils with 1 μM formyl-methionyl-leucyl-phenylalanine (fMLP) in the presence of cytochalasin B (5 μg/mL) evoked the release of 21.2% and 19.8% of lysozyme and β-glucuronidase, respectively, of the initial cellular content. As shown in Table 1, furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole (**12**) was inactive (IC_{50} value of >30 μM) while its 5-phenyl derivative **13**, with an IC_{50} value of 16.3 and 11.4 μM against lysozyme and β-glucuronidase release, respectively, was comparable to the positive trifluoperazine. The same potency was observed for 5-furan derivative **16** with an IC_{50} value of 19.5 and 11.3 μM against lysozyme and β-glucuronidase release, respectively. An electron-withdrawing NO_2 substituted on 5-phenyl or 5-furanyl group led to the devoid of activity as in the cases of **14** and **17**. Among them, compound **15** exhibited significant inhibitory effects, with an IC_{50} value of 7.4 and 5.0 μM against lysozyme and β-glucuronidase release, respectively.



Scheme 2. Reagents and conditions: (a) $(CH_2O)_n$, NH_4OAc, AcOH, MW@100 W, 0.5 h; (b) substituted-benzaldehyde, NH_4OAc, AcOH, MW@100 W, 0.5 h; (c) furaldehyde or 5-nitro-2-furaldehyde diacetate, NH_4OAc, AcOH, MW@100 W, 0.5 h; (d) 3-(5-nitro-2-furyl)acrolein NH_4OAc, AcOH, MW@100 W, 0.5 h.

Table 1
The inhibitory effects of furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole derivatives on the release of β-glucuronidase and lysozyme from rat neutrophils[a,b]

Compd	R	Neutrophil degranulation (IC_{50}, μM)	
		Lysozyme	β-Glucuronidase
12	—	>30.0	>30.0
13	H	16.3 ± 5.0	11.4 ± 0.5
14	NO_2	>10.0	>10.0
15	CHO	7.4 ± 1.4	5.0 ± 0.6
16	H	19.5 ± 7.3	11.3 ± 0.6
17	NO_2	>30.0	>30.0
18	—	>30.0	>30.0
	Trifluoperazine	11.9 ± 2.8	12.5 ± 1.6

[a] Values are means ± SE of at least three separate experiments.
[b] Induced by fMLP (0.3 μM)/cytochalasin B (5 μg/ml).

3.2. Neutrophil superoxide generation

fMLP (0.3 μM)/CB (5 μg/mL) or phorbol myristate (PMA; 3 nM) stimulated superoxide anion generation in rat neutrophils. As shown in Table 2, none of them was active in the inhibition of PMA-induced superoxide anion generation.

3.3. Lipopolysaccharide (LPS)-induced nitric oxide production

Treatment of Raw 264.7 murine macrophage cells with LPS (1 μg/mL) for 24 h induced NO production as assessed by measuring the accumulation of nitrite, a stable metabolite of NO, in the media based on Griess reaction.[22] Results from Table 2 indicated the tetracyclic furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole (**12**) itself was inactive. A phenyl or a substituted phenyl moiety appended on the imidazole ring such as compounds **13–15** were also inactive while the furan derivative **16** showed a marginal inhibitory activity, with an IC_{50} value of 11.9 μM. Compound **17** was approximately eightfold more active than **16** indicated that an electron-withdrawing substituent such as NO_2 is crucial for the suppression LPS-induced NO production. Insertion of a conjugated double bond did not affect the inhibitory activity in which both **17** and **18** were able to suppress LPS-induced NO production in a dose-dependent manner, with an IC_{50} value of 1.5 and 1.3 μM, respectively, which are more active than that of the positive *N*-(3-(aminomethyl)benzyl)acetamidine (1400 W).[23,24] To ensure that these compounds did not interfere with the survival of the macrophages, Raw 264.7 cells were treated with **16–18**, respectively, for 24 h. Cell viability was determined by methylene blue assay[25] and the results have been shown in Figure 2. Compounds **16–18** were non-cytotoxic at the concentration up to10 μM.

3.4. Nitric oxide (NO)-scavenging effects

NO-scavenging effects of compounds **16–18** were measured according to the reported method.[26] Sodium nitroprusside (SNP) (2.5 mM) was incubated alone or in combination with tested compound (10 μM) or 1400 W (10 μM) in light at room temperature for 60 min. Results of Figure 3 indicated the co-incubation of SNP with compounds **16–18** or 1400 W did not affect the levels of nitrite.

3.5. Lipopolysaccharide (LPS)-induced iNOS protein expression

To clarify the possible mechanism of involved in furo[3′,2′:3,4]-naphtho[1,2-*d*]imidazole derivatives **16–18**, we examined the inhibitory effects of these derivatives, on the protein expression of iNOS in Raw 264.7 cells.[25] Results from Figure 4 indicated the unstimulated cells expressed negligible levels of iNOS protein whereas LPS-induced cells expressed the protein in high levels. Compounds **16–18** exhibited more potent inhibitory activities than that of the positive 1400 W (IC_{50} = 2.7 μM).[24] These compounds were capable of inhibiting LPS-induced iNOS protein expression at a dose-dependent manner in which compound **18** exhibited the most potent inhibitory effect. At a concentration of 5.0 μM, expression of iNOS proteins was almost completely inhibited by compound **18** while the positive control 1400 W demonstrated only a moderate

Table 2
The inhibitory effects of furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole derivatives on the accumulation of nitric oxide in the culture media of RAW 264.7 cells in response to LPS and on superoxide anion generation in rat neutrophils stimulated with fMLP/CB or PMA[a,b]

Compd	R	Nitric oxide production (IC_{50}, μM)	Neutrophil superoxide generation (IC_{50}, μM)	
		RAW 264.7	fMLP	PMA
12	—	>30.0	>30.0	>30.0
13	H	>10.0	>30.0	>30.0
14	NO_2	>10.0	>30.0	>30.0
15	CHO	>10.0	>30.0	>30.0
16	H	11.9 ± 3.1	>30.0	>30.0
17	NO_2	1.5 ± 0.3	>30.0	>30.0
18	—	1.3 ± 0.1	>30.0	>30.0
1400 W[c]		2.7 ± 0.1	—	—
DPI[d]		—	5.2 ± 0.8	4.3 ± 1.1

[a] Values are means ± SE of at least three separate experiments.
[b] When compounds exhibited cytotoxicity at 30 μM, a lower concentration was applied.
[c] 1400 W: *N*-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide.
[d] DPI: diphenylene iodonium chloride.

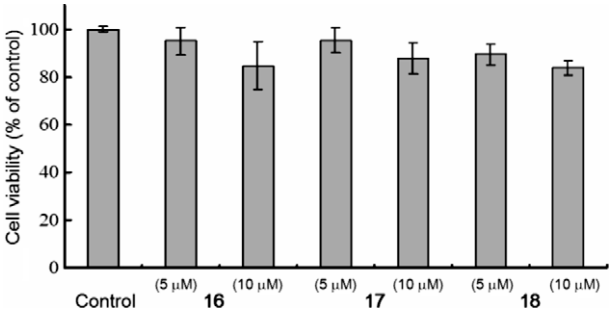


Figure 2. Effects of **16**–**18** on the cell viability of a Raw 264.7 murine macrophage cell line. Cells were treated with various concentrations of compounds for 24 h. Cell viability was determined by methylene blue assay.

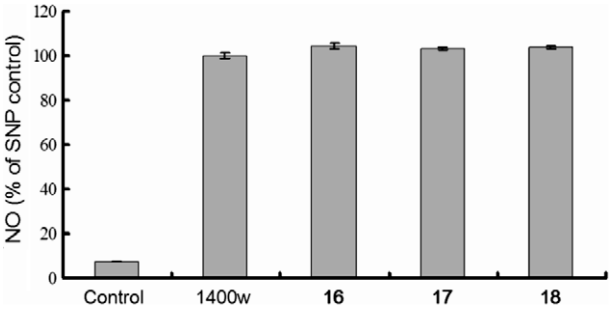


Figure 3. NO-scavenging effects of compounds **16**–**18**. SNP solution (2.5 mM) in PBS was incubated alone or with tested compound (10 μM) or 1400 W (10 μM) in the presence of light at room temperature for 60 min.

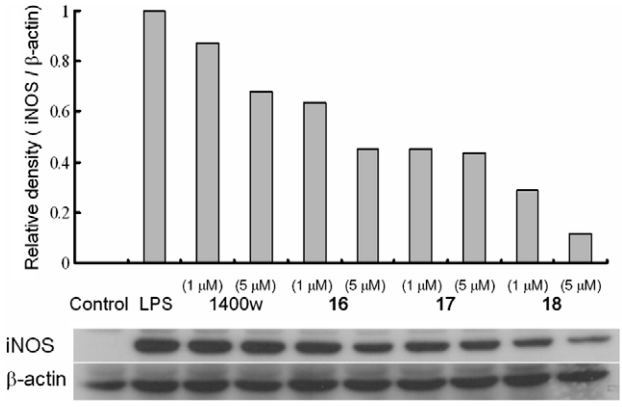


Figure 4. Inhibitory effects of 1400 W and compounds **16**–**18** on LPS-induced iNOS protein expression. Cells (5×10^5 cells/well) were pretreated with the indicated concentrations of tested compounds for 2 h before incubation with 0.1 μg/mL LPS for 24 h. Proteins (15 μg) of total cell lysates were separated by 8% SDS–PAGE, iNOS protein expression was detected by western blot and analyzed by densitometry. The relative density was calculated as the ratio of iNOS expression to β-actin expression.

inhibitory activity. These results are in agreement with their inhibitory effects on NO production (Table 2). Thus, the results of NO inhibition may be directly mediated by the down regulation of iNOS protein expression. Further experiments were carried out to compare the inhibitory potencies of 1400 W and **18** on the expression of iNOS. Results indicated that compound **18**, with an IC_{50} of 0.52 μM in the inhibition of iNOS expression, is approximately fivefold more potent than that of the positive 1400 W.

3.6. Induction of lung iNOS mRNA expression in CLP rat animal model

In order to understand the pathophysiology of sepsis, much research has been directed toward animal models. One such model is the cecal ligation and puncture (CLP) and this model mimics the features of sepsis in humans.[27–29] In order to further confirm the anti-inflammatory effect of compound **18** in vivo, we have also examined the inhibitory effect of **18** on the iNOS mRNA expression in CLP rat lung tissue. As shown in Figure 5, approximately 30% of iNOS mRNA expression was inhibited by the peritoneal injection of **18** 1 h before the surgery. Our results also indicated **18** exhibited a significant anti-inflammatory effect in vivo and was more effective than the positive hydrocortisone (H-Cort).

3.7. Inhibitory activity of compound 18 on the production of prostaglandin E2 (PGE2) in CLP rat animal model

The inhibitory effect of **18** on the PGE2 production in rat serum was investigated and the PGE2 concentration was analyzed by ELISA. Results from Figure 6 indicated that the sepsis-induced PGE2 production in rat serum decreased 150% by the pretreatment of **18** in a dose of 10 mg/kg.

4. Conclusion

We have identified certain furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole derivatives as potential anti-inflammatory agents. Among them, 2-(4-formylphenyl)furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole (**15**) was more active than the positive trifluoperazine against lysozyme and β-glucuronidase release. (*E*)-2-(2-(5-Nitrofuran-2-yl)vinyl)furo-[3′,2′:3,4]naphtho[1,2-*d*]imidazole (**18**), with an IC_{50} of 0.52 μM in the inhibition of iNOS expression, is approximately fivefold more potent than that of the positive 1400 W. In CLP rat animal model, **18** inhibited sepsis-induced iNOS mRNA expression in rat lung tissue and PGE2 production in rat serum. Therefore, furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole derivatives could be developed as a novel type of anti-inflammatory agents on the ground that NO and PGE2 are important mediators for the induction of inflammatory responses. Further structural optimizations are ongoing.

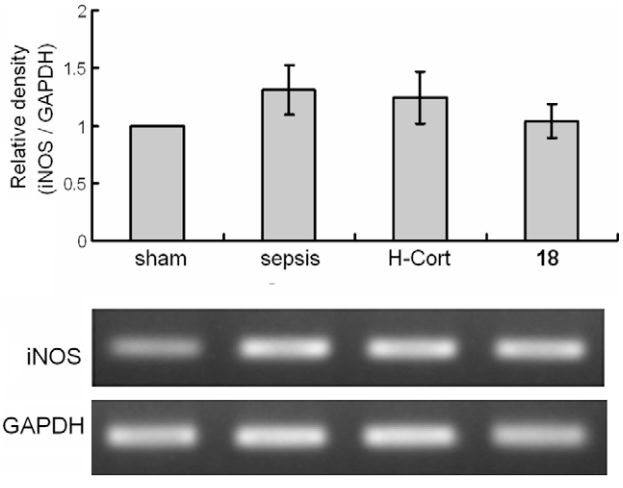


Figure 5. Inhibitory effects of compound **18** on the iNOS mRNA expression in CLP rat animal model. Six to eight weeks of Sprague-Dawley rats were pretreated with H-Cort (10 mg/kg) or **18** (10 mg/kg) by peritoneal injection 1 h before the surgery. Lung tissue sample was collected 24 h after the surgery. Total RNA was extracted for the synthesis of first strand cDNA. The iNOS mRNA expression was analyzed by PCR amplification.

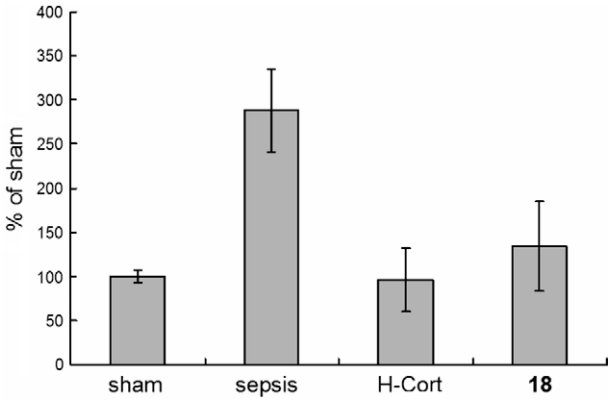


Figure 6. Inhibitory effects of compound **18** on the PGE2 expression in CLP rat animal model. Six to eight weeks of Sprague-Dawley rats were pretreated with H-Cort (10 mg/kg) or **18** (10 mg/kg) by peritoneal injection 1 h before the surgery. Serum sample was collected 24 h after the surgery. The PGE2 in the serum were analyzed by the PGE2 ELISA (R&D, USA). Data shown are the average of three independent studies.

5. Experimental

5.1. General

TLC: precoated (0.2 mm) Silica Gel 60 F_{254} plates from EM Laboratories, Inc.; detection by UV light (254 nm). Mp: Electrothermal IA9100 digital melting-point apparatus; uncorrected. ^1H and ^{13}C NMR spectra: Varian-Unity-400 spectrometer at 400 and 100 MHz or Varian-Gemini-200 spectrometer at 200 and 50 MHz, chemical shifts δ in ppm with $SiMe_4$ as an internal standard (=0 ppm), coupling constants *J* in Hz. Elemental analyses were carried out on a Heraeus CHN-O-Rapid elemental analyzer, and results were within ±0.4% of calculated values.

5.1.1. Naphtho[1,2-*b*]furan-4,5-dione (10) and Naphtho[2,3-*b*]furan-4,9-dione (11)

A mixture of 2-hydroxy-*p*-napthoquinone (2.61 g, 15 mmol), KI (0.50 g) and 40% chloroacetaldehyde (5.20 mL, 6.28 g, 32 mmol) in 1.0 N KOH (20 mL) was heated at reflux for 4 h (TLC monitoring). After cooling, the resulting precipitate was collected, washed with H_2O, purified by column chromatography (hexane/CH_2Cl_2 1:1) and recrystallized from MeOH to give 1.19 g (40%) of **10** as a red solid. Mp: 211–212 °C (lit. 213–215 °C[20]). ^1H NMR (400 MHz, $CDCl_3$): 6.88 (d, 1H, *J* = 2.0 Hz, 3-H), 7.48 (m, 1H, 7-H), 7.52 (d, 1H, *J* = 2.0 Hz, 2-H), 7.67 (m, 1H, 8-H), 7.73 (m, 1H, 9-H), 8.09 (m, 1H, 6-H). ^{13}C NMR (100 MHz, $CDCl_3$): 108.85, 121.46, 122.35, 128.38, 128.68, 130.30, 130.56, 135.44, 145.07, 160.55, 174.45, 180.47. Anal. calcd for $C_{12}H_6O_3$: C, 72.73; H, 3.05. Found: C, 72.61; H, 3.07. Compound **11** was obtain 0.30 g (10%) as a yellow solid; mp: 227–228 °C (lit. 218–220 °C[20]). ^1H NMR (400 MHz, $CDCl_3$): 7.01 (d, 1H, *J* = 1.6 Hz, 3-H) 7.74–7.79 (m, 3H, 2-, 6-, and 7-H), 8.19–8.25 (m, 2H, 5- and 8-H). ^{13}C NMR (100 MHz, $CDCl_3$): 108.65, 126.95, 127.07, 130.49, 132.47, 133.23, 133.87, 133.95, 148.62, 152.72, 173.60, 180.56. Anal. calcd for $C_{12}H_6O_3$: C, 72.73; H, 3.05. Found: C, 72.58; H, 3.05.

5.1.2. Furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole (12)

To a suspension of **2** (0.20 g, 1.0 mmol) in acetic acid (30 mL) was added ammonia acetate (0.23 g, 3.0 mmol) and paraformaldehyde (0.3 g). The reaction mixture was heated with stirring under microwave irradiation (100 W) for 30 min (TLC monitoring). The solvent was removed in vacuo and the residue suspended in H_2O (20 mL). The resulting precipitate was purified by column chromatography (MeOH:CH_2Cl_2 1/50) and recrystallized from EtOH to give **4** as a white solid (0.13 g, 61%). Mp: 282–283 °C. ^{1}H NMR (400 MHz, DMSO-d_6 + TFA-*d*): 7.32 (d, 1H, *J* = 2.0 Hz, 4-H), 7.75 (m, 2H, 8- and 9-H), 8.25 (d, 1H, *J* = 2.0 Hz, 5-H), 8.34 (m, 1H, 7-H), 8.52 (m, 1H, 10-H), 9.68 (s, 1H, 2-H). ^{13}C NMR (100 MHz, DMSO-d_6 + TFA-*d*): 105.62, 111.05, 118.79, 119.62, 121.10, 122.42, 122.54, 123.62, 127.36, 127.67, 137.45, 146.90, 149.35. EIMS (*m*/*z*): 208 (M^+), 197, 153, 126. Anal. calcd for $C_{13}H_8N_2O$: C, 74.99; H, 3.87; N, 13.45. Found: C, 75.04; H, 3.89; N, 13.50.

5.1.3. 2-Phenylfuro[3′,2′:3,4]naphtho[1,2-*d*]imidazole (13)

From **10** and benzaldehyde as described for **12**, to give a gray solid (0.18 g, 65% yield). Mp: 313–314 °C. ^{1}H NMR (400 MHz, DMSO-d_6 + TFA-*d*): 7.27 (d, 1H, *J* = 2.4 Hz, 4-H), 7.66–7.77 (m, 5-H, Ar-H), 8.20–8.26 (m, 4H, Ar-H), 8.55 (m, 1H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6 + TFA-*d*): 105.56, 111.07, 118.53, 119.48, 121.04, 122.60, 123.46, 123.48, 124.49, 127.17, 127.56, 128.04 (2C), 129.92 (2C), 133.14, 146.38, 146.88, 149.35. EIMS (*m*/*z*): 302 (M^+), 273, 153, 126. Anal. calcd for $C_{19}H_{12}N_2O$: C, 80.27; H, 4.25; N, 9.85. Found: C, 79.97; H, 4.35; N, 9.73.

5.1.4. 2-(4-Nitrophenyl)furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole (14)

From **10** and 4-nitrobenzaldehyde as described for **12**, to give a brown solid (0.18 g, 56% yield). Mp: 304–305 °C. ^{1}H NMR (400 MHz, DMSO-d_6 + TFA-*d*): 7.30 (d, 1H, *J* = 2.0 Hz, 4-H), 7.72 (m, 2-H, 8- and 9-H), 8.23 (d, 1H, *J* = 2.0 Hz, 5-H), 8.30 (m, 1H, 7-H), 8.43 (m, 2H, Ar-H), 8.52 (m, 2H, Ar-H), 8.58 (m, 1H, 10-H). ^{13}C NMR (100 MHz, DMSO-d_6 + TFA-*d*): 105.73, 111.71, 119.26, 119.79, 121.20, 122.82, 124.95 (2C), 125.44, 126.59, 127.22, 127.64, 128.80 (2C), 130.54, 144.54, 146.88, 149.31, 149.54. EIMS (*m*/*z*): 329 (M^+), 283, 255, 228. Anal. calcd for $C_{19}H_{11}N_3O_3 \cdot 1.2H_2O$: C, 65.01; H, 3.86; N, 11.98. Found: C, 64.97; H, 3.84; N, 12.05.

5.1.5. 2-(4-Formylphenyl)furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole (15)

From **10** and terephthalaldehyde as described for **12**, to give a brown solid (0.22 g, 72% yield). Mp: 253–254 °C. ^{1}H NMR (400 MHz, DMSO-d_6 + TFA-*d*): 7.32 (d, 1H, *J* = 2.0 Hz, 4-H), 7.74 (m, 2-H, 8- and 9-H), 8.23 (m, 2H, Ar-H), 8.25 (d, 1H, *J* = 2.0 Hz, 5-H), 8.31 (m, 1H, 7-H), 8.42 (m, 2H, Ar-H), 8.61 (m, 1H, 10-H), 10.12 (s, 1H, CHO). ^{13}C NMR (100 MHz, DMSO-d_6 + TFA-*d*): 105.68, 111.37, 118.87, 119.72, 121.16, 122.79, 124.54, 125.61, 127.30, 127.74, 128.44 (2C), 128.81, 130.61 (2C), 138.53, 145.20, 146.97, 149.54, 192.62. Anal. calcd for $C_{20}H_{12}N_2O_2 \cdot 1.0H_2O$: C, 72.71; H, 4.28; N, 8.48. Found: C, 72.56; H, 4.36; N, 8.09.

5.1.6. 2-Furanylfuro[3′,2′:3,4]naphtho[1,2-*d*]imidazole (16)

From **10** and 2-furaldehyde as described for **12**, to give a white solid (0.21 g, 75% yield). Mp: 270–271 °C. ^{1}H NMR (400 MHz, DMSO-d_6 + TFA-*d*): 6.97 (dd, *J* = 3.6, 1.6 Hz, 1H, furanyl-4′-H), 7.23 (d, *J* = 2.0 Hz, 1H, 4-H), 7.65–7.70 (m, 3H, furanyl-3′-, 8- and 9-H), 8.17 (d, *J* = 2.0 Hz, 1H, 5-H), 8.24 (m, 1H, 7-H), 8.26 (dd, *J* = 1.6, 0.8 Hz, 1H, furanyl-5′-H), 8.46 (m, 1H, 10-H). ^{13}C NMR (100 MHz, DMSO-d_6 + TFA-*d*): 105.57, 111.16, 113.93, 116.61, 118.55, 119.56, 121.12, 122.56, 123.19, 124.15, 127.20, 127.52, 137.58, 139.21, 146.79, 148.22, 149.52. Anal. calcd for $C_{17}H_{10}N_2O_2$: C, 74.44; H, 3.67; N, 10.21. Found: C, 74.12; H, 3.75; N, 10.06.

5.1.7. 2-(5-Nitrofuran-2-yl)furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole (17)

From **10** and 5-nitro-2-furaldehyde diacetate as described for **12**, to give a red solid (0.26 g, 82% yield). Mp: 314–315 °C. ^{1}H NMR (400 MHz, DMSO-d_6 + TFA-*d*): 7.17 (d, 1H, *J* = 2.0 Hz, 4-H), 7.61 (m, 2H, 8- and 9-H), 7.63 (d, *J* = 4.0 Hz, 1H, furanyl-3′-H), 7.82 (d, *J* = 4.0 Hz, 1H, furanyl-4′-H), 8.06 (d, *J* = 2.0 Hz, 1H, 5-H), 8.18 (m, 1H, 7-H), 8.40 (m, 1H, 10-H). ^{13}C NMR (100 MHz, DMSO-d_6 + TFA-*d*): 106.14, 112.12, 115.30, 116.33, 119.84, 120.37, 121.52, 123.18, 126.12, 127.36, 127.58, 127.85, 136.06, 143.08, 146.75, 150.20, 153.25. Anal. calcd for $C_{17}H_9N_3O_4 \cdot 0.4$EtOH: C, 63.31; H, 3.40; N, 12.44. Found: C, 63.47; H, 3.21; N, 12.21.

5.1.8. (*E*)-2-(2-(5-Nitrofuran-2-yl)vinyl)furo[3′,2′:3,4]naphtho[1,2-*d*]imidazole (18)

From **10** and 3-(5-nitro-2-furyl)acrolein as described for **12**, to give a brown solid (0.25 g, 71% yield). Mp: 284–285 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 7.17 (d, *J* = 4.0 Hz, 1H, furanyl-3′-H), 7.29 (d, 1H, *J* = 2.0 Hz, 4-H), 7.42 (d, 1H, *J* = 16.0 Hz, =CH), 7.58 (d, 1H, *J* = 16.0 Hz, =CH), 7.65 (m, 2H, 8- and 9-H), 7.80 (d, *J* = 4.0 Hz, 1H, furanyl-4′-H), 8.22 (d, *J* = 2.0 Hz, 1H, 5-H), 8.30 (m, 1H, 7-H), 8.45 (m, 1H, 10-H). ^{13}C NMR (100 MHz, DMSO-d_6 + TFA-*d*): 105.77, 111.25, 113.37, 115.10, 117.42, 118.63, 120.20, 121.58, 122.72, 124.22, 125.16, 125.29, 127.73, 128.20, 143.80, 147.19, 150.24, 152.80, 152.90. Anal. calcd for $C_{19}H_{11}N_3O_4 \cdot 1.3H_2O$: C, 61.88; H, 3.72; N, 11.40. Found: C, 61.66; H, 3.83; N, 11.36.

5.2. Cell culture

Raw 264.7 cells, purchased from American Type Culture Collection, USA, were cultured in DMEM, supplemented with 5% fetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 2 mM L-glutamine, and 1 mM non-essential amino acids in a 10-cm plate at a density of 1×10^6 cells/mL, at 37 °C in a humidified atmosphere containing 5% CO_2.

5.2.1. Neutrophil superoxide generation[30]

Compound stock solution (30 mM in DMSO) was prepared and stored at −25 °C, and was diluted with DMSO to 1–20 mM range at room temperature before experiment. The final percentage of DMSO in the reaction mixture was less than 0.5% (v/v). Rat (Sprague-Dawley) peripheral blood neutrophils[31] were isolated and incubated with test compounds for 5 min at 37 °C before stimulation with 1 μM formyl-Met-Leu-Phe (fMLP) for another 45 min, respectively. Neutrophils were assessed by the determination of β-glucuronidase and lysozyme, in the supernatant.[32] In the superoxide anion generation experiments, neutrophils were stimulated with fMLP (0.3 μM)/CB (5 μg/mL) or phorbol myristate (PMA; 3 nM) for 30 min in the presence of cytochrome *c*, and the superoxide anion generation was measured in terms of superoxide dismutase-inhibitable cytochrome *c* reduction.[33,34]

5.2.2. Nitrite measurement

Nitrite was measured by adding 100 μL of the Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μL of medium for 5 min. The optical density at 550 nm (OD_{550}) was measured with a microplate reader. Concentrations were calculated by comparison with the OD_{550} of a standard solution of sodium nitrite prepared in culture medium.

5.2.3. Cell viability

To evaluate whether the suppressive effect of compounds **16–18** on NO production is related to cell viability, Raw 264.7 cells were treated with LPS for 24 h, and washed with 500 μL of phosphate buffer solution (PBS) before resuspended in 1 mL of culture

medium. Cell viability was assessed by the methylene blue dye staining method. Briefly, cells at 2×10^5 cells/well were seeded into 24-well microtiter plates and treated with various reagents for 24 h. Cells were stained with 1% methylene blue for 40 min, then the plates were washed with tap water three times following addition of 1% sacosyl for 4 h. Finally, the plates were read immediately on an enzyme-linked immunosorbent microplate reader (ELISA Biokinetics Reader) at a wavelength of 570 nm.[35] Live cells treated with LPS in the absence of compounds **16–18** were set as 100%. Percentages of relative survival in the presence of vehicle or various concentrations of compounds **16–18** were compared against each other.

5.2.4. NO-scavenging activity

To understand the inhibitory effects of compounds **16–18** on NO production, sodium nitroprusside (SNP) was freshly prepared at 5 mM in PBS was incubated alone or in combination with 10 μM of the different compounds. SNP is an inorganic complex where NO is found as NO^+ and light irradiation is necessary for the release of NO. Therefore, incubation mixtures were incubated in light at room temperature, and nitrite levels were determined after 60 min by Griess reaction.

5.2.5. Western blotting

The effects of compounds **16–18** on iNOS protein expression in LPS stimulated Raw 264.7 cell were assessed by western blot. Briefly, Raw 264.7 cells were pretreated with tested compound for 2 h before stimulation in the presence or absence of LPS (0.1 μg/mL). Cell were harvested and lysed with RIPA lysis buffer (50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% NP-40, 1% sodiumdeoxycholate, 1 mM phenylmethyl-sulfonylfluoride, 1 mM EDTA, no added proteinase) 24 h after treatment. The total protein in the cell lysates was assayed according to the method described by Bradford.[36] Equal amounts (15 μg) of protein were separated using 8% SDS–PAGE and analyzed by western blot (ECL Amersham). The relative expression of proteins was quantified densitometrically using the software LabWorks 4.5 and was calculated according to the reference bands of β-actin.

5.3. Animal model of sepsis

Sprague-Dawley rats six to eight weeks old, weight 150–200 g were anesthetized with citosol (10 mg/100 g) before induction of sepsis. The abdomen was shaved and washed with 10% povidone iodine. Cecal ligation and puncture (CLP) was performed by three punctures with 18G syringes. Three to four animals were grouped randomly for each experimental group. All groups were pretreated by peritoneal injection of hydrocortisone (H-Cort) (10 mg/kg) or compound **18** (10 mg/kg) 1 h before surgery. Sham operation animals were underwent same procedures as the experimental animals. All animals were resuscitated with 1 mL of normal saline immediately after the surgery. Twenty-four hours after the surgery, animals were re-anesthetized; blood and tissue samples were collected for further analysis. All experiments were performed according to the National Science Council guideline in Taiwan. The procedure of the animal model was approved by animal facility care in Kaohsiung Medical University, Taiwan.

5.3.1. RNA extraction and determination of iNOS mRNA expression

Total RNA was extracted from lung samples by Trizol-Reagent (Invirogen) according to the manufactures manual. One microgram of RNA was used for the reverse transcription of first strand cDNA using the oligo dT primers. Five microliters of cDNA were used for the amplification of iNOS and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA expression. The primers for rat iNOS are as follows: 5′-ACT GCG TCG CTT CAT TAG GT-3′ (forward) and 5′-TAG GCA AGC GCT TTA CCA CT-3′ (reverse). The primers for rat glyceraldehyde 3-phosphate dehydrogenase are as follows, forward: 5′-ATG GCA CAG TCA AGG CTG AGA-3′ reverse: 5′-AGA CGC CAG TAG ACT CCA CGA C-3′. The amplicons were electrophoresed on a 3% agarose gel. The gel was then examined and band intensities were measured using a spectrophotometer (Lab work) and normalized against GAPDH.

5.3.2. Measurement of serum prostaglandin E2 (PGE2) concentration

Blood samples were collected separately from animal portal ventral vein and immediately put into tubes. The blood samples were centrifuged at 3000 rpm for 20 min, and the serum was decanted and stored at −70 °C. The PGE2 in the serum was analyzed by the PGE2 ELISA (R&D, USA).

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