A New Quinolone and Other Constituents from the Fruits of *Tetradium ruticarpum*: Effects on Neutrophil Pro-Inflammatory Responses

by **Tzu-Ying Wang**[a]), **Jin-Bin Wu***[a]), **Tsong-Long Hwang**[b]), **Yueh-Hsiung Kuo**[c]), and **Jih-Jung Chen***[d])

[a]) Graduate Institute of Pharmaceutical Chemistry, College of Pharmacy, China Medical University, Taichung 404, Taiwan

[b]) Graduate Institute of Natural Products, Chang Gung University, Taoyuan 333, Taiwan

[c]) Institute of Chinese Pharmaceutical Sciences, College of Pharmacy, China Medical University, Taichung 404, Taiwan

[d]) Graduate Institute of Pharmaceutical Technology, Tajen University, Pingtung 907, Taiwan (phone: +886-8-7624002 (ext. 2827); fax: +886-8-7625308; e-mail: jjchen@mail.tajen.edu.tw)

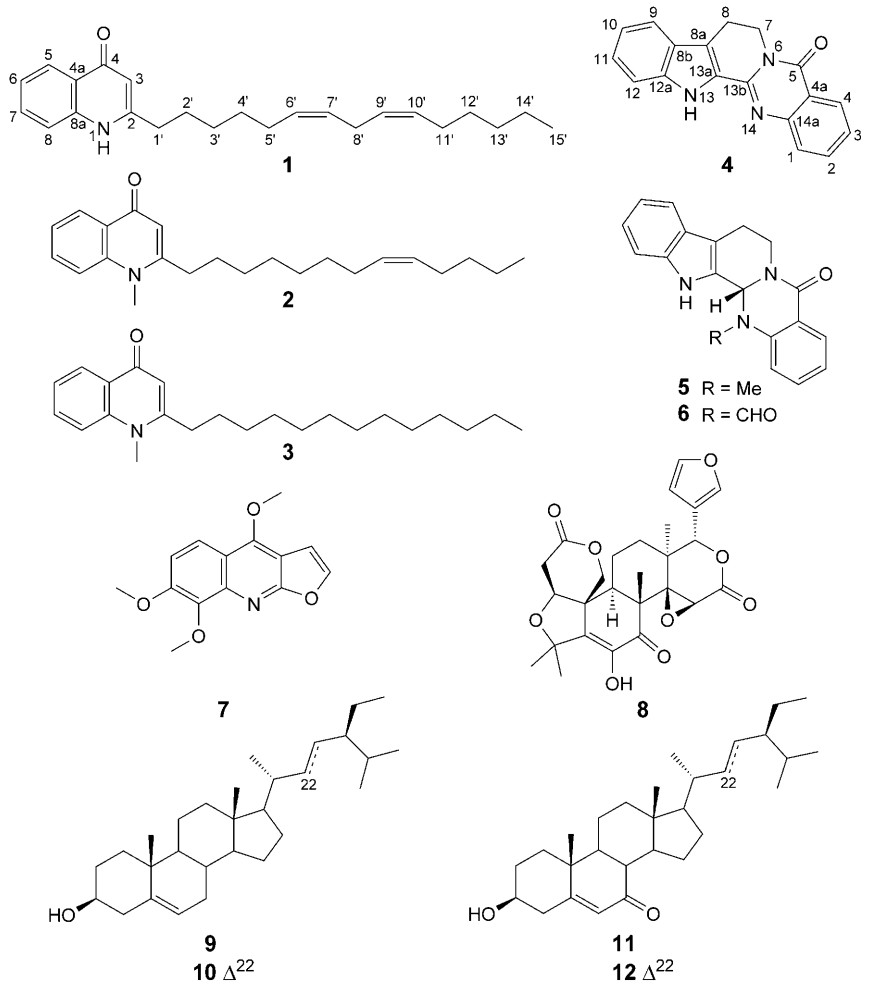
The fruit of *Tetradium ruticarpum* is widely used in healthcare products for the improvement of blood circulation, headache, abdominal pain, amenorrhea, chill limbs, migraine, and nausea. A new quinolone, 2-[(6*Z*,9*Z*)-pentadeca-6,9-dienyl]quinolin-4(1*H*)-one (**1**), has been isolated from the fruits of *T. ruticarpum*, together with eleven known compounds. The structure of the new compound was determined by NMR and MS analyses. Rutaecarpine (**4**), evodiamine (**5**), and skimmianine (**7**) exhibited inhibition ($IC_{50} \leq 20.9$ μM) of $O_2^{\cdot-}$ generation by human neutrophils in response to *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B (fMLP/CB). In addition, **1**, evocarpine (**2**), **4**, **7**, and evodol (**8**) inhibited fMLP/CB-induced elastase release with IC_{50} values ≤ 14.4 μM.

Introduction. – *Tetradium ruticarpum* (A. JUSS.) T. HARTLEY (synonym: *Evodia rutaecarpa* (JUSS.) BENTH; Rutaceae) is a shrub or small tree, distributed in forests at middle altitudes from the Himalaya eastward to Taiwan [1]. A decoction of its fruit has been used as Chinese traditional drug (*Wu-Chu-Yu*) in the treatment of headache, abdominal pain, dysentery, postpartum haemorrhage, amenorrhea, chill limbs, and nausea [2]. Previous studies of this plant have led to the isolation of indole alkaloids [3][4], quinolone alkaloids [5][6], limonoids [7], and their derivatives. Many of these compounds exhibit cytotoxic [8], antiallergic [9], and anti-inflammatory [10] activities, and show inhibitory activities against DNA topoisomerase [8], diacylglycerol acyltransferase [11], and nuclear factor of activated T cells [12].

As part of our studies on the anti-inflammatory constituents of Formosan plants, many species have been screened for *in vitro* inhibitory activity of neutrophil pro-inflammatory responses. In the course of this screening, a CH_2Cl_2-soluble fraction of the fruit of *T. ruticarpum* proved to be active. Investigation of the active fraction afforded a new quinolone, 2-[(6*Z*,9*Z*)-pentadeca-6,9-dienyl]quinolin-4(1*H*)-one (**1**), and eleven known compounds. This article describes the structural elucidation of **1**, and the inhibitory activity of the isolates on $O_2^{\cdot-}$ generation and elastase release by neutrophils.

Results and Discussion. – *Structure Elucidation.* Extensive chromatographic purification of the CH_2Cl_2-soluble fraction of the MeOH extract of fruits of *T.*

ruticarpum on a silica-gel column and preparative TLC afforded a new, **1**, and eleven known compounds, **2**–**12**. The new compound **1** was isolated as white amorphous powder. Its molecular formula, $C_{24}H_{33}NO$, was determined on the basis of the *quasi*-molecular ion at *m*/*z* 374.2462 ($[M+Na]^+$, $C_{24}H_{33}NNaO^+$; calc. 374.2460) in the HR-ESI-MS spectrum (positive-ion mode) and was supported by the ^{1}H-, ^{13}C-, and DEPT-NMR data. The presence of the NH and CO groups in the molecule was revealed by the bands at 3125 and 1637 cm^{-1}, respectively, in the IR spectrum, which was confirmed by signals at δ(H) 10.85 (br. *s*, D_2O exchangeable, NH) and δ(C) 178.8 (C=O) in the ^{1}H- and ^{13}C-NMR spectra.



Comparison of the ^{1}H-NMR data (*Table 1*) of **1** with those of 1-methyl-2-[(6*Z*,9*Z*)-pentadeca-6,9-dienyl]quinol-4(1*H*)-one (MPQ) [5] suggested that their structures were closely related, except that the NH group (δ(H) 10.85, br. *s*, D_2O exchangeable) of **1** replaced the MeN group of MPQ. This was supported by both HMBC correlations

between NH (δ(H) 10.85) and C(3) (δ(C) 108.1), C(8) (δ(C) 118.6), and C(1′) (δ(C) 34.3), and NOESY correlations between NH (δ(H) 10.85) and both H–C(8) (δ(H) 7.73) and H–C(1′) (δ(H) 2.70).

Table 1. *^{1}H-NMR Data of MPQ and* **1**. At 400 MHz in $CDCl_3$; δ in ppm, J in Hz.

Position	MPQ[a])	**1**		
	δ(H)	δ(H)	NOE	HMBC[b])
H–C(3)	6.15 (*s*)	6.25 (*s*)	1′	4, 4a, 1′
H–C(5)	8.44 (*dd*, *J*=8.0, 2.0)	8.36 (*dd*, *J*=8.0, 1.2)	6	4, 7, 8a
H–C(6)	7.25–7.8 (*m*)	7.33 (br. *t*, *J*=8.0)	5, 7	4a, 8
H–C(7)	7.25–7.8 (*m*)	7.58 (*td*, *J*=8.0, 1.2)	6, 8	5, 8a
H–C(8)	7.25–7.8 (*m*)	7.73 (br. *d*, *J*=8.0)	7, NH	4a, 6, 8a
CH_2(1′)	2.70 (*m*)[c])	2.70 (*t*, *J*=7.6)	3, 2′, NH	2, 3, 2′, 3′
CH_2(2′)	1.40 (br. *m*)[c])	1.65–1.79 (*m*)	1′, 3′	2, 4′
CH_2(3′)	1.40 (br. *m*)[c])	1.20–1.45 (*m*)	2′	1′, 5′
CH_2(4′)	1.40 (br. *m*)[c])	1.20–1.45 (*m*)	5′	2′, 5′, 6′
CH_2(5′)	2.08 (br. *m*)[c])	2.04 (*m*)[c])	4′, 6′, 8′	3′, 6′, 7′
H–C(6′)	5.40 (*m*)[c])	5.34 (*m*)[c])	5′	4′, 7′, 8′
H–C(7′)	5.40 (*m*)[c])	5.34 (*m*)[c])	8′	5′, 6′, 9′
CH_2(8′)	2.70 (*m*)[c])	2.77 (br. *t*, *J*=6.4)	5′, 7′, 9′, 11′	6′, 9′, 10′
H–C(9′)	5.40 (*m*)[c])	5.34 (*m*)[c])	8′	7′, 8′, 11′
H–C(10′)	5.40 (*m*)[c])	5.34 (*m*)[c])	11′	8′, 12′
CH_2(11′)	2.08 (br. *m*)[c])	2.04 (*m*)[c])	8′, 10′, 12′	9′, 10′, 13′
CH_2(12′)	1.40 (br. *m*)[c])	1.20–1.45 (*m*)	11′	10′, 14′
CH_2(13′)	1.40 (br. *m*)[c])	1.20–1.45 (*m*)		11′, 15′
CH_2(14′)	1.40 (br. *m*)[c])	1.20–1.45 (*m*)	15′	12′, 13′, 15′
Me(15′)	0.90 (br. *t*, *J*=6.0)	0.88 (br. *t*, *J*=7.2)	14′	13′, 14′
NH		10.85 (br. *s*)	8, 1′	3, 8, 1′
MeN	3.69 (*s*)			

[a]) MPQ = 1-Methyl-2-[(6*Z*,9*Z*)-pentadeca-6,9-dienyl]quinol-4(1*H*)-one [5]. [b]) From the H- to the C-atom. [c]) Center of overlapping signals.

According to the above data, the structure of **1** was elucidated as 2-[(6*Z*,9*Z*)-pentadeca-6,9-dienyl]quinolin-4(1*H*)-one. This structure was supported by ^{1}H,^{1}H-COSY and NOESY experiments (*Table 1*), and ^{13}C-NMR assignments were confirmed by DEPT, HSQC, and HMBC techniques (*Table 1*).

The known isolates were readily identified by comparison of their physical and spectroscopic data (UV, IR, ^{1}H-NMR, $[\alpha]_D$, and MS) with those of the corresponding authentic samples or literature values. They include two quinolone alkaloids, evocarpine (**2**) [5] and dihydroevocarpine (**3**) [5], three indolopyridoquinazoline alkaloids, rutaecarpine (**4**) [13], evodiamine (**5**) [14], and 14-formyldihydrorutaecarpine (**6**) [15], a furoquinoline alkaloid, skimmianine (**7**) [16], a limonoid, evodol (**8**) [5], and four steroids, a mixture of β-sitosterol (**9**) [17] and stigmasterol (**10**) [17], and a mixture of 3β-hydroxystigmast-5-en-7-one (**11**) [18][19] and 3β-hydroxystigmasta-5,22-dien-7-one (**12**) [20]. Among the known isolates, compounds **7** and **9**–**12** have been found for the first time in this plant species.

Biological Studies. Neutrophils accumulate at sites of inflammation and immunological reactions in response to locally existing chemotactic mediators. The bacterial *N*-formyl peptides, such as *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), are some of the first identified and most potent chemoattractants for neutrophils [21]. In this study, the effects on neutrophil pro-inflammatory responses of the compounds isolated from the fruits of *T. ruticarpum* were evaluated by measuring the suppression of fMLP/CB-induced $O_2^{\bullet-}$ generation and elastase release by human neutrophils. The inhibitory-activity data on neutrophil pro-inflammatory responses are shown in *Table 2*. The phosphatidylinositol-3-kinase inhibitor LY294002 (*Sigma*, St. Louis, MO, USA) was used as a positive control for $O_2^{\bullet-}$ generation and elastase release [22][23]. From the results of our biological tests, the following conclusions can be drawn: *i*) compounds **4**, **5**, and **7** exhibited inhibitory activities on human neutrophil $O_2^{\bullet-}$ generation ($IC_{50} \leq 20.9$ μM); *ii*) compounds **1**, **2**, **4**, **7**, and **8** inhibited fMLP/CB-induced elastase release with IC_{50} values ≤ 14.4 μM; *iii*) compounds **1** and **2**, both possessing an unsaturated long-chain alkyl group, showed strong inhibition against fMLP-induced elastase release, whereas their analogue, dihydroevocarpine (**3**), with a saturated long-chain alkyl group, was only weakly active; *iv*) among the indolopyridoquinazoline analogues **4**–**6**, rutaecarpine (**4**), with a C(13b)=N(14) bond, exhibited more effective inhibition than its analogues **5** (with a MeN group) and **6** (with an *N*-formyl group) against fMLP-induced $O_2^{\bullet-}$ generation and elastase release; *v*) compound **4** was the most effective among these compounds, with an IC_{50} value of 12.1 ± 1.6 μM against fMLP-induced $O_2^{\bullet-}$ generation; *vi*) evocarpine (**2**) exhibited the most effective inhibition among the isolates, with an IC_{50} value of 2.6 ± 0.26 μM, against fMLP-induced elastase release; and *vii*) the steroids **9**–**12** were inactive.

Table 2. *Inhibitory Effects of* **1**–**12** *on* $O_2^{\bullet-}$ *Generation and Elastase Release by Human Neutrophils in Response to* N-*formyl*-L-*methionyl*-L-*leucyl*-L-*phenylalanine*/*Cytochalasin B*

Compound	IC_{50} [μM][a])	
	$O_2^{\bullet-}$ Generation	Elastase Release
1	>50	3.1 ± 0.68
2	>50	2.6 ± 0.26
3	>50	49.5 ± 8.6
4	12.1 ± 1.6	6.9 ± 0.83
5	20.6 ± 3.8	49.2 ± 5.1
6	>50	48.8 ± 3.8
7	20.9 ± 3.5	14.4 ± 1.3
8	>50	11.7 ± 0.6
Mixture of **9** and **10**	>50	>50
Mixture of **11** and **12**	>50	>50
LY294002[b])	1.1 ± 0.12	1.9 ± 0.23

[a]) Concentration necessary for 50% inhibition; results are presented as means ± S.E.M. ($n=4$). [b]) LY294002: Phosphatidylinositol-3-kinase inhibitor, used as a positive control for the $O_2^{\bullet-}$ generation and elastase release.

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Experimental Part

General. Column chromatography (CC): silica gel *60* (SiO_2; 70–230 or 230–400 mesh; *Merck*). TLC: silica gel *60* F_{254} precoated plates (*Merck*). Optical rotations: *Jasco DIP-370* polarimeter; in $CHCl_3$ or MeOH. UV Spectra: *Jasco UV-240* spectrophotometer; δ_{max} (log ε) in nm. IR Spectra: *Perkin-Elmer 2000* FT-IR spectrophotometer; $\tilde{\nu}$ in cm^{-1}. ^1H-, ^{13}C-, and 2D-NMR Spectra: *Varian Unity-Plus-400* spectrometer; δ in ppm rel. to Me_4Si, *J* in Hz. EI-MS: *VG-Biotech Quatro-5022* mass spectrometer; in *m/z* (rel. %). ESI- and HR-ESI-MS: *Bruker APEX-II* mass spectrometer; in *m/z*.

Plant Material. The fruits of *T. ruticarpum* were bought from the *Guanghan Trade Limited Company*, Kaohsiung, Taiwan, in July 2008 and identified by Prof. *J.-J. C.* A voucher specimen (68050) was deposited with the Herbarium of the Department of Forest Resource, Management and Technology, National Pingtung University of Science and Technology, Pingtung, Taiwan.

Extraction and Isolation. The dried fruits (5.1 kg) of *T. ruticarpum* were extracted with MeOH (3 × 20 l) for 3 d. The extract was concentrated under reduced pressure at 35°, and the residue (495 g) was partitioned between CH_2Cl_2 and H_2O (1:1) to provide the CH_2Cl_2-soluble fraction (*Fr. A*; 105 g). The H_2O-soluble fraction was further extracted with BuOH, and the BuOH-soluble part (*Fr. B*; 186 g) and the H_2O-soluble one (*Fr. C*; 206 g) were separated. *Fr. A* (105 g) was purified by CC (SiO_2, 4.0 kg), 70–230 mesh; CH_2Cl_2/MeOH gradient) to afford 13 fractions: *Fr. A1* (eluted with 8 l of CH_2Cl_2), *Fr. A2* (with 6 l of CH_2Cl_2/MeOH 100:1), *Fr. A3* (with 5 l of CH_2Cl_2/MeOH 95:1), *Fr. A4* (with 6 l of CH_2Cl_2/MeOH 90:1), *Fr. A5* (with 6 l of CH_2Cl_2/MeOH 80:1), *Fr. A6* (with 6 l of CH_2Cl_2/MeOH 70:1), *Fr. A7* (with 5 l of CH_2Cl_2/MeOH 50:1), *Fr. A8* (with 5 l of CH_2Cl_2/MeOH 30:1), *Fr. A9* (with 5 l of CH_2Cl_2/MeOH 10:1), *Fr. A10* (with 5 l of CH_2Cl_2/MeOH 5:1), *Fr. A11* (with 6 l of CH_2Cl_2/MeOH 2:1), *Fr. A12* (with 6 l of CH_2Cl_2/MeOH 1:1), and *Fr. A13* (with 7 l of MeOH). *Fr. A2* (5.7 g) was subjected to CC (SiO_2, 230–400 mesh (245 g); hexane/acetone 10:1, 1.2-l fractions) to give nine subfractions: *Frs. A2.1–A2.9*. *Fr. A2.5* (195 mg) was further purified by prep. TLC (SiO_2; hexane/acetone 5:1) to yield a mixture of **9** and **10** (35.5 mg; R_f 0.38). *Fr. A4* (6.4 g) was purified by CC (SiO_2, 230–400 mesh (258 g); hexane/acetone 10:1, 1.4-l fractions) to give ten subfractions: *Frs. A4.1–A4.10*. *Fr. A4.9* (188 mg) was further purified by prep. TLC (SiO_2; hexane/AcOEt 4:1) to afford **4** (7.2 mg; R_f 0.63). *Fr. A6* (8.5 g) was subjected to CC (SiO_2, 230–400 mesh (365 g); hexane/AcOEt 5:1, 1.0-l fractions) to give nine subfractions: *Frs. A6.1–A6.9*. *Fr. A6.7* (850 mg) was further purified by CC (SiO_2, 230–400 mesh (32 g); hexane/acetone 3:1, 80-ml fractions) to give eight subfractions: *Frs. A6.7.1–A6.7.8*. *Fr. A6.7.2* (95 mg) was further purified by prep. TLC (SiO_2; $CHCl_3$/AcOEt 20:1) to yield a mixture of **11** and **12** (4.6 mg; R_f 0.17). *Fr. A6.7.8* (90 mg) was further purified by prep. TLC (SiO_2; CH_2Cl_2/acetone 30:1) to provide **5** (8.9 mg; R_f 0.65). *Fr. A6.8* (870 mg) was further purified by CC (SiO_2, 230–400 mesh (33 g); CH_2Cl_2/acetone 30:1, 85-ml fractions) to give eight subfractions: *Frs. A6.8.1–A6.8.8*. *Fr. A6.8.5* (102 mg) was further purified by prep. TLC (SiO_2; $CHCl_3$/AcOEt 20:1) to yield **6** (3.5 mg; R_f 0.45). *Fr. A6.9* (1.2 g) was further purified by CC (SiO_2, 230–400 mesh (43 g); hexane/acetone 3:1, 250-ml fractions) to give seven subfractions: *Frs. A6.9.1–A6.9.7*. *Fr. A6.9.6* (205 mg) was further purified by prep. TLC (SiO_2; hexane/AcOEt 2:1) to afford **7** (4.7 mg; R_f 0.29). *Fr. A8* (5.5 g) was subjected to CC (SiO_2, 230–400 mesh (235 g); hexane/AcOEt 2:1, 200-ml fractions) to give nine subfractions: *Frs. A8.1–A8.9*. *Fr. A8.8* (650 mg) was further purified by CC (SiO_2, 230–400 mesh (28 g); hexane/acetone 3:1, 75-ml fractions) to give eight subfractions: *Frs. A8.8.1–A8.8.8*. *Fr. A8.8.6* (82 mg) was further purified by prep. TLC (SiO_2; hexane/acetone 2:1) to afford **3** (3.4 mg; R_f 0.55). *Fr. A8.8.8* (94 mg) was further purified by prep. TLC (SiO_2; hexane/AcOEt 1:1) to provide **8** (3.2 mg; R_f 0.48). *Fr. A10* (10.2 g) was subjected to CC (SiO_2, 230–400 mesh (455 g); hexane/acetone 2:1, 850-ml fractions) to give twelve subfractions: *Frs. A10.1–A10.12*. *Fr. A10.3* (202 mg) was further purified by prep. TLC (SiO_2; hexane/acetone 2:1) to yield **2** (6.8 mg; R_f 0.44). *Fr. A10.7* (198 mg) was further purified by prep. TLC (SiO_2; $CHCl_3$/MeOH 30:1) to afford **1** (5.1 mg; R_f 0.50).

*2-[(6Z,9Z)-Pentadeca-6,9-dienyl]quinolin-4(1*H*)-one* (**1**). White amorphous powder. UV (MeOH): 213 (4.43), 236 (4.45), 314 (4.11), 327 (4.10). IR (KBr): 3125 (NH), 1637 (CO). ^1H-NMR: see *Table 1*.

^{13}C-NMR (100 MHz, $CDCl_3$): 14.0 (C(15′)); 22.8 (C(14′)); 25.6 (C(8′)); 26.9 (C(5′)); 27.1 (C(11′)); 28.5 (C(2′)); 28.8 (C(3′)); 29.1 (C(4′)); 29.2 (C(12′)); 31.4 (C(13′)); 34.3 (C(1′)); 108.1 (C(3)); 118.6 (C(8)); 123.6 (C(6)); 124.9 (C(4a)); 125.3 (C(5)); 127.5 (C(7′)); 128.2 (C(9′)); 129.4 (C(6′)); 130.1 (C(10′)); 131.7 (C(7)); 140.7 (C(8a)); 155.4 (C(2)); 178.8 (C(4)). ESI-MS: 374 ($[M+Na]^+$). HR-ESI-MS: 374.2462 ($[M+Na]^+$, $C_{24}H_{33}NNaO^+$; calc. 374.2460).

Biological Assay. The effect of the isolated compounds on the neutrophil pro-inflammatory response was evaluated by monitoring the inhibition of $O_2^{\cdot-}$ generation and the release of elastase in fMLP/CB-activated human neutrophils in a concentration-dependent manner.

Preparation of Human Neutrophils. Human neutrophils from venous blood of healthy, adult volunteers (20–28 years old) were isolated using a standard method of dextran sedimentation prior to centrifugation in a *Ficoll-Hypaque* gradient and hypotonic lysis of erythrocytes [24]. Purified neutrophils containing >98% viable cells, as determined by the trypan-blue exclusion method [25], were re-suspended in a Ca^{2+}-free *Hank*'s buffered salt soln. (HBSS) at pH 7.4 and were maintained at 4° prior to use.

Measurement of $O_2^{\cdot-}$ *Generation.* Measurement of $O_2^{\cdot-}$ generation was based on the SOD-inhibitable reduction of ferricytochrome *c* [26][27]. In brief, after supplementation with 0.5 mg/ml ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils (6×10^5/ml) were equilibrated at 37° for 2 min and incubated with different concentrations of compounds or DMSO (as control) for 5 min. Cells were incubated with cytochalasin B (CB; 1 μg/ml) for 3 min prior to the activation with 100 nM *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) for 10 min. Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (*Hitachi U-3010*, Tokyo, Japan). Calculations were based on the differences in the reactions with and without SOD (100 U/ml) divided by the extinction coefficient for the reduction of ferricytochrome *c* ($\varepsilon=21.1$ mM^{-1} cm^{-1}).

Measurement of Elastase Release. Degranulation of azurophilic granules was determined by measuring the elastase release as described previously [27]. Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide as the elastase substrate. Briefly, after supplementation with MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (100 μM), neutrophils (6×10^5/ml) were equilibrated at 37° for 2 min and incubated with compounds for 5 min. Cells were stimulated with fMLP (100 nM)/CB (0.5 μg/ml), and changes in the absorbance at 405 nm were monitored continuously in order to measure the elastase release. The results were expressed as the percent of elastase release in the fMLP/CB-activated, drug-free control system.

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