Bioorganic & Medicinal Chemistry 17 (2009) 7313–7323

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Hepatoprotective amide constituents from the fruit of *Piper chaba*: Structural requirements, mode of action, and new amides

Hisashi Matsuda [a], Kiyofumi Ninomiya [a,b], Toshio Morikawa [a,b], Daisuke Yasuda [a], Itadaki Yamaguchi [a], Masayuki Yoshikawa [a,*]

[a] Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607-8412, Japan
[b] Pharmaceutical Research and Technology Institute, Kinki University, 3-4-1 Kowakae, Higashi-osaka, Osaka 577-8502, Japan

ARTICLE INFO

Article history:
Received 3 July 2009
Revised 18 August 2009
Accepted 19 August 2009
Available online 29 August 2009

Keywords:
Hepatoprotective activity
Piperine
Piper chaba
Piperaceae
Piperchabamide
Anti-TNF-α activity

ABSTRACT

The 80% aqueous acetone extract from the fruit of *Piper chaba* (Piperaceae) was found to have hepatoprotective effects on D-galactosamine (D-GalN)/lipopolysaccharide-induced liver injury in mice. From the ethyl acetate-soluble fraction, three new amides, piperchabamides E, G, and H, 33 amides, and four aromatic constituents were isolated. Among the isolates, several amide constituents inhibited D-GalN/tumor necrosis factor-α (TNF-α)-induced death of hepatocytes, and the following structural requirements were suggested: (i) the amide moiety is essential for potent activity; and (ii) the 1,9-decadiene structure between the benzene ring and the amide moiety tended to enhance the activity. Moreover, a principal constituent, piperine, exhibited strong in vivo hepatoprotective effects at doses of 5 and 10 mg/kg, po and its mode of action was suggested to depend on the reduced sensitivity of hepatocytes to TNF-α.

1. Introduction

Piper chaba HUNTER (syn. *P. retrofractum* VAHL., Piperaceae) is widely distributed in Southeast Asia. The fruit of this plant is commonly called 'Dee Plee' in Thailand and has been used as an antiflatulent, expectorant, antitussive, antifungal, uterus-contracting agent, sedative-hypnotic, appetizer, and counter-irritant in the traditional medicine of Thailand.[1] In the course of our characterization studies on this natural medicine,[2–4] we previously reported that 40 constituents were isolated from the 80% aqueous acetone extract of the fruit of *P. chaba*. The aqueous acetone extract and the isolates were found to show protective effects on ethanol- or indomethacin-induced gastric lesions in rats,[2] inhibitory effects on the increase in serum aspartate aminotransaminase (sAST) and alanine aminotransaminase (sALT) levels induced by D-galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced liver injury in mice and on cell death induced by D-GalN/tumor necrosis factor-α (TNF-α) in primary cultured mouse hepatocytes,[3] and promoting effects on adipogenesis of 3T3-L1 cells.[4] As a continuing study of the constituents from the fruit of *P. chaba*, we additionally isolated three new amide constituents named piperchabamides E (**1**), G (**2**), and H (**3**). This paper deals with the isolation and structural elucidation of the three new amides (**1**–**3**) from the fruit of *P. chaba* as well as the hepatoprotective effects and structural requirements of the amide constituents for the reduced sensitivity to TNF-α.[3,5]

2. Results and discussion

2.1. Protective effects of the aqueous acetone extract from the fruit of *P. chaba* on liver injury induced by D-GalN/LPS in mice

The dried fruit of *P. chaba* (purchased in Thailand) was extracted with 80% (v/v) aqueous acetone at room temperature to yield an aqueous acetone extract (19.7% from the dried fruit).[2] As shown in Table 1, the 80% aqueous acetone extract from the fruit of *P. chaba* showed inhibitory effects on the increase in sAST and sALT, as markers of liver injury, induced by D-GalN/LPS in mice at doses of 25–50 mg/kg, po.

2.2. Effects of the aqueous acetone extract from the fruit of *P. chaba* on D-GalN or D-GalN/TNF-α-induced cytotoxicity in primary cultured mouse hepatocytes

Infection with hepatitis C virus and chronic consumption of alcohol are major causes of liver injury, cirrhosis, and hepatocellular carcinoma worldwide. TNF-α is known to mediate a number of forms of organ injury through its induction of cellular apoptosis. In

* Corresponding author. Tel.: +81 75 595 4633; fax: +81 75 595 4768.
E-mail address: myoshika@mb.kyoto-phu.ac.jp (M. Yoshikawa).

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doi:10.1016/j.bmc.2009.08.050

Table 1
Inhibitory effects of the 80% aqueous acetone extract from the fruit of *P. chaba* on D-GalN/LPS-induced liver injury in mice

Treatment	Dose (mg/kg, po)	*n*	sAST		sALT	
			(Karmen unit)	Inhibition (%)	(Karmen unit)	Inhibition (%)
Normal (vehicle)	—	7	86 ± 5[a]	—	28 ± 6[a]	—
Control (D-GalN/LPS)	—	9	5016 ± 560	—	6373 ± 928	—
80% Aqueous acetone ext.	25	8	2598 ± 616[a]	49.0	3692 ± 1301	42.3
	50	8	1422 ± 437[a]	72.9	1448 ± 524[a]	77.6
	100	8	577 ± 35[a]	90.0	304 ± 12[a]	95.7

Each value represents the mean ± SEM.
Significantly different from the control.
[a] p <0.01.

the liver, the biological effects of TNF-α have been implicated in hepatic injury induced by hepatic toxins, ischemia/reperfusion, viral hepatitis, and alcohol.[6–8] Therefore, TNF-α is considered to be an important target in research to discover anti-inflammatory and hepatoprotective agents.

D-GalN/LPS-induced liver injury is recognized to develop via immunological responses.[9] This type of liver injury occurs in two forms. First, depletion of uridine triphosphate and increased sensitivity to TNF-α in hepatocytes is induced by D-GalN. Second, pro-inflammatory mediators, such as TNF-α, are released from LPS-activated macrophages (Kupffer's cells). Apoptosis of hepatocytes by TNF-α is reported to have an important role in D-GalN/LPS-induced liver injury.[10]

In our previous study on hepatoprotective compounds from natural medicines, we reported that several constituents from *Hovenia dulcis*,[11] *Bupleurum scorzonerifolium*,[12,13] *Curcuma zedoaria*,[14–16] *Angelica furcijuga*,[17,18] *Betula platyphylla* var. *japonica*,[19] *Pisum sativum*,[20] *Salacia reticulata*,[21] *Tilia argentea*,[22] *Anastatica hierochuntica*,[23] *Panax notoginseng*,[24] *Cyperus longus*,[25] *Erycibe expansa*,[26] *Camellia sinensis*,[27] *Sedum sarmentosum*,[28,29] *Sinocrassula indica*,[30] and *Hedychium coronarium*[31] showed hepatoprotective effects on liver injury induced by D-GalN/LPS in mice and/or D-GalN-induced cytotoxicity in primary cultured hepatocytes. Since the aqueous acetone extract from the fruit of *P. chaba* showed hepatoprotective effects on D-GalN/LPS-induced liver injury in mice (vide ante), the inhibitory effects on D-GalN or D-GalN/TNF-α-induced cytotoxicity in primary cultured mouse hepatocytes were examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Table 2, the aqueous acetone extract of this herbal medicine inhibited the cytotoxicity induced by both D-GalN and D-GalN/TNF-α in hepatocytes (IC_{50} value = 18 and 11 μg/mL, respectively).

2.3. Chemical constituents from the fruit of *P. chaba*

The 80% aqueous acetone extract from the fruit of *P. chaba* was partitioned between EtOAc–H_2O (1:1, v/v) to give an EtOAc-soluble fraction (9.7%) and an aqueous phase. The EtOAc-soluble fraction significantly inhibited the D-GalN and D-GalN/TNF-α-induced cytotoxicity in hepatocytes [inhibition (%) at 30 μg/mL: 94 ± 2 (p <0.01) and 70 ± 6 (p <0.01), respectively]. The EtOAc-soluble fraction was subjected to SiO_2 and ODS column chromatographies and finally to HPLC (ODS column, eluted with MeOH–H_2O or CH_3CN–H_2O solvent system) to furnish three new amide constituents, piperchabamides E[3] (**1**, 0.0046%), G (**2**, 0.0064%), and H (**3**, 0.0085%) together with four aromatic compounds, benzenepropanoic acid[32] (0.13%), 3,4-methylendioxycinnamaldehyde[33] (0.0019%), piperonal[3,32] (**4**, 0.0047%), and methyl piperate[2,34,35] (**5**, 0.11%), and 33 amide compounds, piperchabamide A[2] (**6**, 0.0029%), *N*-cinnamoylpiperidine[36] (**7**, 0.0016%), ilepcimide[37] (**8**, 0.0048%), $\Delta^{\alpha,\beta}$-dihydropiperine [= piperanine[2,38,39] (**9**, 0.42%)], piperine[2,32,35,40,41] (**10**, 2.95%), isopiperine[41] (**11**, 0.056%), isochavicine[41] (**12**, 0.039%), chavicine[41] (**13**, 0.0042%), piperoleine B[3,40] (**14**, 0.0060%), pipernonaline[2,42] (**15**, 0.47%), piperchabamide B[2] (**16**, 0.0041%), dehydropipernonaline[2,43] (**17**, 0.056%), piperundecalidine[3,42] (**18**, 0.017%), piperchabamide C[2] (**19**, 0.0032%), fragaramide[44] (**20**, 0.0006%), 5,6-dihydropiperlonguminine[3,39] (**21**, 0.015%), piperlonguminine[2,35] (**22**, 0.072%), retrofractamide C[3,45] (**23**, 0.036%), piperchabamide D[2] (**24**, 0.0037%), dihydroguineensine[46] (**25**, 0.0015%), retrofractamide A[3,40,47] (**26**, 0.0075%), pipercide [= retrofractamide B[2,40,42,47] (**27**, 0.049%)], guineensine[2,35,40,42,47] (**28**, 0.081%), brachystamide B[3,48] (**29**, 0.012%), *N*-isobutyl-(2*E*,4*E*)-deca-2,4-dienamide[3,49,50] (**30**, 0.018%), *N*-isobutyl-(2*E*,4*E*)-dodeca-2,4-dienamide[3,49,50] (**31**, 0.0034%), *N*-isobutyl-(2*E*,4*E*)-tetradeca-2,4-dienamide[50] (**32**, 0.0044%), *N*-isobutyl-(2*E*,4*E*)-hexadeca-2,4-dienamide[51] (**33**, 0.011%), *N*-isobutyl-(2*E*,4*E*)-octadeca-2,4-dienamide[2,51] (**34**, 0.23%), *N*-isobutyl-(2*E*,4*E*,8*Z*)-tetradeca-2,4,8-trienamide[52] (**35**, 0.0017%), *N*-isobutyl-(2*E*,4*E*,12*Z*)-octadeca-2,4,12-trienamide[53] (**36**, 0.0057%), *N*-isobutyl-(2*E*,4*E*,14*Z*)-eicosa-2,4,14-trienamide[2,53] (**37**, 0.18%), and piperic acid isopentyl amide[54] (**38**, 0.0011%).

2.4. Structures of piperchabamides E (1), G (2), and H (3)

Piperchabamide E (**1**) was obtained as colorless oil with positive optical rotation ($[\alpha]_D^{24}$ +18.7 in MeOH). Its IR spectrum showed absorption bands at 2963, 1646, 1545, 1506, 1489, 1446, 1257, and 1238 cm^{-1} ascribable to methylene, an unsaturated amide group, and an aromatic ring. The EIMS of **1** showed a molecular

Table 2
Inhibitory effects of the 80% aqueous acetone extract from the fruit of *P. chaba* on D-GalN or D-GalN/TNF-α-induced cytotoxicity in primary cultured mouse hepatocytes

	Inhibition (%)					IC_{50} (μg/mL)
	0 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	
D-GalN-induced (hepatocytes)						
80% Aqueous acetone ext.	0 ± 2	7 ± 3	17 ± 1[a]	76 ± 2[a]	−22 ± 5[b]	18
D-GalN/TNF-α-induced (hepatocytes)						
80% Aqueous acetone ext.	0 ± 1	22 ± 2[a]	57 ± 4[a]	66 ± 4[a]	105 ± 9[a]	11

Each value represents the mean ± SEM (N = 4).
Significantly different from the control.
[a] p <0.01.
[b] Cytotoxic effect was observed.

ion peak at *m/z* 287 (M^+), and the molecular formula was determined to be $C_{17}H_{21}NO_3$ by high-resolution EIMS measurement. The ^1H and ^{13}C NMR spectra of **1** ($CDCl_3$, Tables 3 and 4) showed signals assignable to two methyls [δ 0.91 (3H, dd, J = 6.9, 7.6 Hz, H_3-4′), 0.91 (3H, d, J = 6.7 Hz, H_3-5′)], two methylenes [δ 1.17, 1.41 (1H each, both m, H_2-3′), 3.15, 3.29 (1H each, both m, H_2-1′)], a methine [δ 1.59 (1H, m, H-2′)], a 3,4-methylenedioxyphenyl group [δ 5.97 (2H, s, H_2-12), 6.75 (1H, d, J = 8.2 Hz, H-10), 6.88 (1H, dd, J = 1.8, 8.2 Hz, H-11), 6.96 (1H, d, J = 1.8 Hz, H-7)], two *trans*-olefinic proton pairs [δ 5.93 (1H, d, J = 15.0 Hz, H-2), 6.65 (1H, dd, J = 10.7, 17.7 Hz, H-4), 6.78 (1H, d, J = 17.7 Hz, H-5), 7.35 (1H, dd, J = 10.7, 15.0 Hz, H-3)], and a conjugated amide group [δ_C 166.2 (C-1)]. The planar structure of **1** was constructed on the basis of various NMR experiments.[55] Specifically, the ^1H–^1H COSY experiments on **1** indicated the presence of three partials written in bold lines, while in the HMBC experiments, long-range correlations were observed between the following proton and carbon pairs: H-2 and C-1; H-5 and C-6; and H_2-1′ and C-1 (Fig. 2). Finally, acid hydrolysis[56] of **1** with 6 M HCl liberated (*R*)-2-methylbutylamine,[57] which was identified by HPLC analysis using the refractive index and optical rotation detectors. On the basis of the above evidence, the stereostructure of **1** was elucidated as shown.

Piperchabamide G (**2**) was isolated as an amorphous powder and the molecular formula, $C_{34}H_{40}N_2O_6$, was determined by EIMS and HREIMS measurements. The ^1H and ^{13}C NMR spectroscopic properties ($CDCl_3$, Tables 3 and 4) of **2** showed signals assignable to two methylenes [δ 1.58, 1.67 (1H each, both m, H_2-4″), 2.40, 2.50 (1H each, both m, H_2-5″)], four methines [δ 2.22 (1H, m, H-3″), 3.60 (1H, br s, H-4), 3.68 (1H, ddd, J = 2.1, 2.1, 9.7 Hz, H-5), 4.05 (1H, dd, J = 9.7, 10.0 Hz, H-2″)], two piperidine rings {δ [1.15 (1H, m), 1.25 (2H, m), 1.43 (1H, m), H-2‴,4‴], 1.43, 1.67 (2H each, both m, H-3′,3‴), 1.58 (4H, m, H-2′,4′), [2.95 (1H, ddd, J = 2.4, 12.5, 12.5 Hz), 3.01 (1H, ddd, J = 3.1, 13.2, 13.2 Hz), 3.48, 3.98 (1H each, both br d, J = ca. 13 Hz), H-1‴,5‴], 3.61, 3.62 (2H each, both br s, H-2′,4′)}, two 3,4-methylenedioxyphenyl groups [δ 5.87, 5.90 (2H each, both br s, H_2-12, 12″), 6.56 (1H, dd, J = 1.5, 7.9 Hz, H-11″), 6.60 (1H, d, J = 1.5 Hz, H-7″), 6.68 (2H, br s, H-7, 11), 6.74 (1H, d, J = 8.2 Hz, H-10), 6.88 (1H, br s, H-10″)], a *cis*-olefin pair [δ 5.71 (1H, ddd, J = 2.1, 5.1, 9.8 Hz, H-3), 5.84 (1H, d, J = 9.8 Hz, H-2)], and two amide groups [δ_C 170.4 (C-1), 173.2 (C-1″)]. As shown in Figure 1, the ^1H–^1H COSY experiment on **2** indicated the presence of partial structures written in bold lines. In the HMBC experiment on **2**, long-range correlations were observed between the following protons and carbons (H-2, H_2-1′,5′ and C-1; H-4, 5 and C-6, 7, 11; H-7, 11 and C-5; H-2″, H_2-1‴, 5‴ and C-1″; H_2-5″ and C-6″, 7″, 11″; H-7″, 11″ and C-5″), as shown in Figure 2. Thus the connectivities of quaternary carbons (C-1, 6, 1″, 6″) in **2** were clarified, and the planar structure of **2** was elucidated.

Piperchabamide H (**3**) was isolated as an amorphous powder. Its molecular formula, $C_{38}H_{46}N_2O_6$, was determined by EIMS and HREIMS measurements. The ^1H and ^{13}C NMR spectra of **3** ($CDCl_3$, Tables 3 and 4) showed signals assignable to four methylenes {δ [1.26 (1H, m), 1.44 (1H, br s), H_2-4″], [1.36 (1H, m), 1.56 (1H, br s), H_2-5″], 1.56 (2H, br s, H_2-6″), 2.13 (2H, m, H_2-7″)}, four methines [δ 2.13, 3.54 (1H each, both br s, H-3″, 4), 3.67 (1H, br d, J = ca. 11 Hz, H-5), 4.02 (1H, dd, J = 10.8, 11.0 Hz, H-2″)], two piperidine rings {δ 1.20, 1.26 (2H each, both m, H_2-2‴,4‴), 1.44 (4H, br s, H_2-3′,3‴), 1.44, 1.56 (2H each both br s, H_2-2′,4′), [3.07 (2H, dd-like), 3.43 (1H, br d, J = ca. 13 Hz), 3.90 (1H, d-like), H_2-1‴,5‴], 3.54 (4H, br s, H_2-1′,5′)}, two 3,4-methylenedioxyphenyl groups [δ 5.87, 5.93 (2H each, both br s, H_2-12, 16″), 6.68 (1H, br s, H-11), 6.73 (1H, d, J = 1.2 Hz, H-7), 6.74 (2H, br d, J = ca. 8 Hz, H-10, 15″), 6.87 (1H, br s, H-11″), 6.88 (1H, br s, H-14″)], a *cis*-olefin pair [δ 5.70 (1H, m, H-3), 5.83 (1H, d, J = 9.8 Hz, H-2)], and two amide groups [δ_C 170.5 (C-1), 173.5 (C-1″)], which were superimposable on those of **2**, except for the signals due to two additional methy-

Table 3
^1H NMR (500 MHz, $CDCl_3$) data on piperchabamides E (**1**), G (**2**), and H (**3**)

Position	**1** δ_H (J Hz)	Position	**2** δ_H (J Hz)	Position	**3** δ_H (J Hz)
2	5.93 (1H, d, 15.0)	2	5.84 (1H, d, 9.8)	2	5.83 (1H, d, 9.8)
3	7.35 (1H, dd, 10.7, 15.0)	3	5.71 (1H, ddd, 2.1, 5.1, 9.8)	3	5.70 (1H, m)
4	6.65 (1H, dd, 10.7, 17.7)	4	3.60 (1H, br s)	4	3.54 (1H, br s)
5	6.78 (d, 17.7)	5	3.68 (1H, ddd, 2.1, 2.1, 9.7)	5	3.67 (1H, br d, ca. 11)
7	6.96 (d, 1.8)	7	6.68 (1H, br s)	7	6.73 (1H, d, 1.2)
10	6.75 (d, 8.2)	10	6.74 (1H, d, 8.2)	10	6.74 (1H, br d, ca. 8)
11	6.88 (dd, 1.8, 8.2)	11	6.68 (1H, br s)	11	6.68 (1H, br s)
12	5.97 (2H, s)	12	5.87 (2H, br s)	12	5.87 (2H, br s)
1′	3.15 (1H, m)	1′,5′	3.61 (2H, br s)	1′,5′	3.54 (4H, br s)
	3.29 (1H, m)		3.62 (2H, br s)	2′,4′	1.44 (2H, br s)
2′	1.59 (1H, m)	2′,4′	1.58 (4H, m)		1.56 (2H, br s)
3′	1.17 (1H, m)	3′	1.43 (1H, m)	3′	1.44 (2H, br s)
	1.41 (1H, m)		1.67 (1H, m)	2″	4.02 (dd, 10.8, 11.0)
4′	0.91 (3H, dd, 6.9, 7.6)	2″	4.05 (1H, dd, 9.7, 10.0)	3″	2.13 (1H, br s)
5′	0.91 (3H, d, 6.7)	3″	2.22 (1H, m)	4″	1.26 (1H, m)
CON*H*	5.66 (1H br s)	4″	1.58 (1H, m)		1.44 (1H, br s)
			1.67 (1H, m)	5″	1.36 (1H, m)
		5″	2.40 (1H, m)		1.56 (1H, br s)
			2.50 (1H, m)	6″	1.56 (2H, br s)
		7″	6.60 (1H, d, 1.5)	7″	2.13 (2H, m)
		10″	6.88 (1H, br s)	8″	5.99 (1H, dt, 15.5, 7.0)
		11″	6.56 (1H, dd, 1.5, 7.9)	9″	6.25 (1H, d, 15.5)
		12″	5.90 (2H, br s)	11″	6.87 (1H, br s)
		1‴,5‴	2.95 (1H, ddd, 2.4, 12.5, 12.5)	14″	6.88 (1H, br s)
			3.01 (1H, ddd, 3.1, 13.2, 13.2)	15″	6.74 (1H, br d, ca. 8)
			3.48 (1H, br d, ca. 13)	16″	5.93 (2H, br s)
			3.98 (1H, br d, ca. 13)	1‴,5‴	3.07 (2H, dd-like)
		2‴,4‴	1.15 (1H, m)		3.43 (1H, br d, ca. 13)
			1.25 (2H, m)		3.90 (1H, d-like)
			1.43 (1H, m)	2‴,4‴	1.20 (2H, m)
		3‴	1.43 (1H, m)		1.26 (2H, m)
			1.67 (1H, m)	3‴	1.44 (2H, br s)

Table 4
^{13}C NMR (125 MHz, $CDCl_3$) data on piperchabamides E (**1**), G (**2**), and H (**3**)

Position	**1** δ_C	Position	**2** δ_C	Position	**3** δ_C
1	166.2	1	170.4	1	170.5
2	123.3	2	134.0	2	133.9
3	140.9	3	123.2	3	123.4
4	124.7	4	38.3	4	37.9
5	138.8	5	46.6	5	46.6
6	130.9	6	137.8	6	137.9
7	105.7	7	108.1	7	108.2
8	148.2	8	147.5	8	147.5
9	148.2	9	146.0	9	146.0
10	108.5	10	108.9	10	108.9
11	122.6	11	121.4	11	121.4
12	101.3	12	100.7	12	100.7
1′	45.3	1′,5′	42.9	1′,5′	42.8
2′	35.1		47.1		47.0
3′	27.1	2′,4′	25.9	2′,4′	25.8
4′	11.3		27.1		25.9
5′	17.2	3′	24.6	3′	24.6
		1″	173.2	1″	173.5
		2″	45.0	2″	45.1
		3″	39.3	3″	39.6
		4″	31.9	4″	29.8
		5″	33.5	5″	29.4
		6″	136.0	6″	27.0
		7″	108.7	7″	32.9
		8″	147.5	8″	129.2
		9″	145.5	9″	129.5
		10″	108.1	10″	132.4
		11″	120.9	11″	105.3
		12″	100.7	12″	147.9
		1‴,5‴	42.9	13″	146.5
			47.0	14″	108.0
		2‴,4‴	26.0	15″	120.2
			27.1	16″	100.9
		3‴	24.6	1‴,5‴	42.8
					47.0
				2‴,4‴	26.1
					26.8
				3‴	24.6

lene groups. The connectivities of the quaternary carbons in **3** were elucidated on the basis of the ^1H–^1H COSY and HMBC experiments, as shown in Figure 2. Next, the stereostructures of **2** and **3** were characterized in nuclear Overhauser enhancement spectroscopy (NOESY) experiments. NOE correlations in **2** and **3** were observed between the following proton pairs (H-2 and H-3; H-3 and H-5; H-5 and H-3″; and H-2″ and H-7, 11) as shown in Figure 3. Consequently, the stereostructures of **2** and **3** were determined to be as shown.

2.5. Effects of the chemical constituents on D-GalN or D-GalN/TNF-α-induced cytotoxicity in primary cultured mouse hepatocytes

Since the aqueous acetone extract of *P. chaba* was found to show protective effects on both D-GalN and D-GalN/TNF-α-induced cytotoxicity in primary cultured mouse hepatocytes (Table 2), the cytoprotective effects of the constituents from this herbal medicine were also examined. As shown in Table 5, piperoleine B (**14**, IC_{50} value = 2.9 μM), *N*-isobutyl-(2*E*,4*E*)-dodeca-2,4-dienamide (**31**, 9.3 μM), and *N*-isobutyl-(2*E*,4*E*,14*Z*)-eicoca-2,4,14-trienamide (**37**, 6.4 μM) showed strong hepatoprotective activity against cytotoxicity induced by D-GalN. Their activities were greater than that of commercial silybin (39 μM),[23–31] which is well known to show potent hepatoprotective effects.[58,59] On the other hand, most of the amide constituents except for brachystamide B (**29**) and **37** significantly inhibited the cell death induced by D-GalN/TNF-α in the hepatocytes at 1–30 μM, as shown in Table 6. Notably, piperchabamide B (**16**, inhibition: 63% at 3 μM), piperlonguminine (**22**, 50%), retrofractamide C (**23**, 51%), piperchabamide D (**24**, 57%), and pipercide (= retrofractamide B, **27**, 54%) showed potent effects at 3 μM with more than 50% inhibition. However, several amide constituents such as **23**, **24**, and **27** exhibited no concentration-dependent inhibition at 3–30 μM, suggesting that they would show cytotoxic effects at higher concentrations. With regard to the structural requirements of the amide constituents for the activity, the amide structure was important [methyl piperate (**5**, inhibition: 21% at 30 μM) < piperine (**10**, 68%)], and **16** and **24**, which have the 1,9-decadiene structure between the benzene ring and amide moiety, tended to show more potent activity.

2.6. Effects of the chemical constituents on NO production in LPS-activated mouse peritoneal macrophages

Next, the effects of the aqueous acetone extract from the fruit of *P. chaba* and its constituents on NO production, as a marker of macrophage activation, in LPS-activated mouse peritoneal macrophages were examined. As shown in Table 7, piperchabamides G (**2**, IC_{50} value = 28 μM), A (**6**, 20 μM), and C (**19**, 42 μM) showed inhibitory effects without cytotoxic effects in the MTT assay, and their inhibitory activities were equivalent to or stronger than that of an NO synthase inhibitor [N^G-monomethyl-L-arginine (L-NMMA), 36 μM].[60,61]

These in vitro findings led us to suggest the following possible mechanisms of action for the hepatoprotective effects of the fruit of *P. chaba*: (i) protection of hepatocytes on D-GalN-induced cytotoxity (**14**, **31**, and **37**), (ii) inhibitory effects on D-GalN/TNF-α-induced cell death in hepatocytes (**16**, **22**–**24**, **27**, etc.), and (iii) inhibitory effects on activation of macrophages induced by LPS (**2**, **6**, and **19**).

2.7. Effects of the chemical constituents on TNF-α-induced cytotoxicity in L929 cells

In order to clarify the effects of the constituents on the sensitivity of hepatocytes to TNF-α, the effects on the TNF-α-induced decrease in cell viability of L929 cells, a TNF-α-sensitive cell line,[62] were examined using the MTT assay. After incubation of L929 cells with 20 pg/mL TNF-α and 0.5 μg/mL actinomycin D for 20 h, the viability of L929 cells without test sample was reduced to ca. 60% compared with that of TNF-α-untreated cells. As shown in Table 8, piperchabamide A (**6**, IC_{50} value = 13 μM), piperine (**10**, 42 μM), piperleine B (**14**, 64 μM), piperchabamides B (**16**, 33 μM) and C (**19**, 42 μM), and *N*-isobutyl-(2*E*,4*E*,14*Z*)-eicoca-2,4,14-trienamide (**37**, 25 μM) inhibited the decrease in the cell viability.

2.8. Protective effects of piperine (10) on liver injury induced by D-GalN/LPS in mice and its mode of action

Finally, we examined the effects of the principal constituent piperine (**10**) on D-GalN/LPS-induced liver injury in mice. As shown in Table 9 and 10 significantly inhibited the increase in sAST and sALT induced by D-GalN/LPS in mice at doses of 5 mg/kg, po. To clarify the mode of action of **10**, its effect on the increase in serum TNF-α levels induced by D-GalN/LPS in mice was examined. As a result, **10** at 5–20 mg/kg, po did not inhibit the increase in serum TNF-α levels apparently different from hydrocortisone (Table 10). Although compound **10** inhibited the death of hepatocytes induced by D-GalN/TNF-α, it could not protect the cells against D-GalN. On the other hand, **10** at 10–100 μM significantly inhibited the death of L929 cells caused by TNF-α, indicating a decrease in the sensitivity of L929 cells to TNF-α (vide ante). These findings suggest a reduction in the sensitivity effect of **10**.[3] Many compounds that inhibit cell death induced

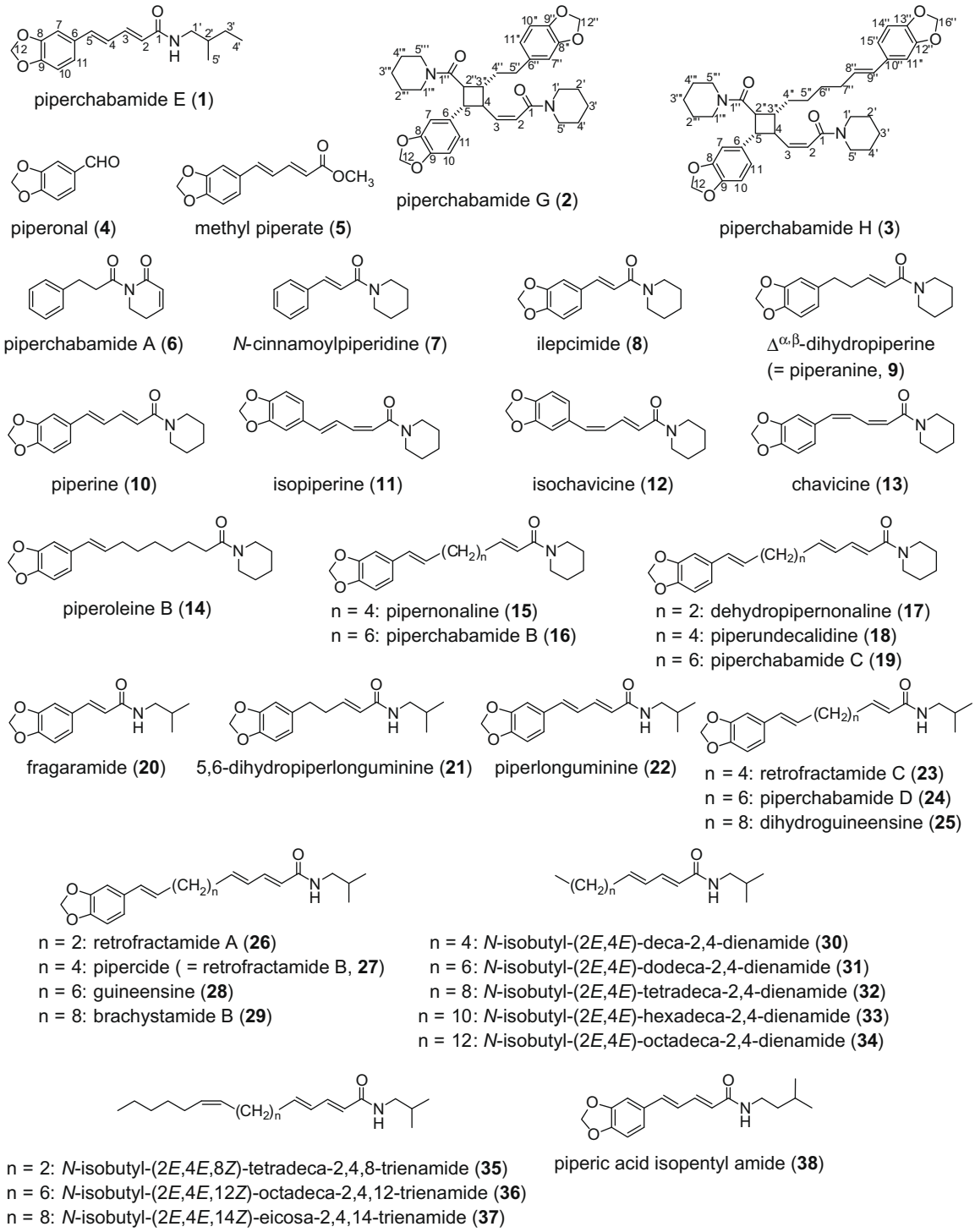

Figure 1. Chemical structures of compounds **1**–**38** from the fruit of *Piper chaba*.

by D-GalN or production of TNF-α have been reported,[14,16,22] but there are few reports on compounds that selectively reduce the sensitivity of hepatocytes to TNF-α.[63,64]

In conclusion, three new amide constituents, piperchabamides E (**1**), G (**2**), and H (**3**), together with 33 known amide constituents and four aromatic constituents, were isolated from the fruit of *P. chaba*. Among the isolates, several constituents were found to show hepatoprotective activities. With regard to the structural requirements, the amide moiety and the 1,9-decadiene structure between the benzene ring and amide moiety were suggested to be important for potent inhibition of the D-GalN/TNF-α-induced death of hepatocytes. Furthermore, a principal amide constituent, piperine (**10**), dose-dependently inhibited the increase in sAST and sALT at doses of 5 and 10 mg/kg po in D-GalN/LPS-treated mice, and this inhibitory effect was suggested to depend on the reduced sensitivity of hepatocytes to TNF-α. The mechanisms of action of the amide constituents should be studied further.

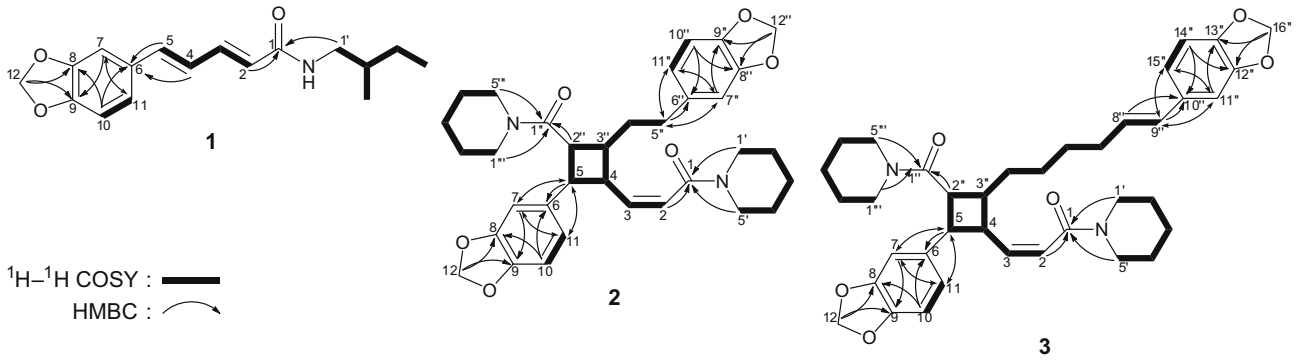

Figure 2. ^{1}H–^{1}H COSY and HMBC correlations of **1**–**3**.

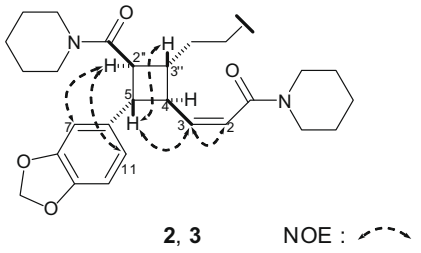

Figure 3. NOESY experiments on **2** and **3**.

3. Experimental

3.1. General

The following instruments were used to obtain spectral and physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l = 5 cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; ^{1}H and ^{13}C NMR spectra, JEOL JNM-LA500 (500 and 125 MHz) and JEOL EX-270 (270 and 68 MHz) spectrometers with tetramethylsilane as an internal standard; EIMS and high-resolution EIMS, JEOL JMS-GCMATE mass spectrometer; HPLC detector, Shimadzu RID-10A refractive index, Shimadzu SPD-10A UV–vis, and Shodex OR-2 optical rotation detectors. Cosmosil 5C_{18}-MS-II (Nacalai Tesque Inc., 250 × 4.6 mm i.d.) and (250 × 20 mm i.d.) columns were used for analytical and preparative HPLC, respectively.

The following experimental conditions were used for chromatography: normal-phase silica gel column chromatography (CC), silica gel BW-200 (Fuji Silysia Chemical, Ltd, 150–350 mesh); reversed-phase silica gel CC, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd, 100–200 mesh); normal-phase TLC, pre-coated TLC plates with silica gel 60F_{254} (Merck, 0.25 mm); reversed-phase TLC, pre-coated TLC plates with silica gel RP-18 F_{254S} (Merck, 0.25 mm); and reversed-phase HPTLC, pre-coated TLC plates with

Table 5
Inhibitory effects of the chemical constituents from the fruit of *P. chaba* on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes

	Inhibition (%)					IC_{50} (μM)
	0 μM	3 μM	10 μM	30 μM	100 μM	
Piperonal (**4**)	0 ± 1	−3 ± 2	0 ± 2	4 ± 3	11 ± 1[b]	
Methyl piperate (**5**)	0 ± 0	−2 ± 2	4 ± 2	5 ± 2	19 ± 3[b]	
$\Delta^{\alpha,\beta}$-Dihydropiperine (= piperanine, **9**)	0 ± 0	5 ± 2	14 ± 2[b]	14 ± 3[b]	11 ± 2[b]	
Piperine (**10**)	0 ± 4	6 ± 6	9 ± 5	2 ± 3	−9 ± 2	
Pipernonaline (**15**)	0 ± 2	−4 ± 3	−6 ± 2	−2 ± 2	−17 ± 3	
Piperundecalidine (**18**)	0 ± 0	−9 ± 2	−12 ± 2	−5 ± 0	−21 ± 2	
5,6-Dihydropiperlonguminine (**21**)	0 ± 0	−4 ± 2	7 ± 3	15 ± 4[a]	5 ± 3	
Piperlonguminine (**22**)	0 ± 0	7 ± 3	13 ± 3[a]	10 ± 3	−14 ± 2	
Retrofractamide C (**23**)	0 ± 0	1 ± 3	1 ± 2	0 ± 4	−18 ± 2	
Retrofractamide A (**26**)	0 ± 3	3 ± 1	7 ± 1	2 ± 1	1 ± 1	
Pipercide (= retrofractamide B, **27**)	0 ± 1	5 ± 8	−6 ± 5	−19 ± 3		
Guineensine (**28**)	0 ± 4	12 ± 4	7 ± 3	6 ± 6	−20 ± 4[c]	
Brachystamide B (**29**)	0 ± 8	10 ± 6	18 ± 3	−17 ± 5	−22 ± 4[c]	
N-Isobutyl-(2*E*,4*E*)-deca-2,4-dienamide (**30**)	0 ± 0	5 ± 1	2 ± 0	4 ± 1	6 ± 1	
N-Isobutyl-(2*E*,4*E*)-dodeca-2,4-dienamide (**31**)	0 ± 3	17 ± 6	41 ± 14[b]	82 ± 6[b]	75 ± 9[b]	9.3
N-Isobutyl-(2*E*,4*E*)-octadeca-2,4-dienamide (**34**)	0 ± 3	2 ± 1	1 ± 1	−1 ± 2	3 ± 2	
N-Isobutyl-(2*E*,4*E*14*Z*)-eicosa-2,4,14-trienamide (**37**)	0 ± 0	32 ± 6[b]	53 ± 2[b]	66 ± 4[b]	89 ± 10[b]	6.4
Silybin[d]	0 ± 2	5 ± 1	8 ± 1	45 ± 9[b]	77 ± 6[b]	39
	0 μM	1 μM	3 μM	10 μM	30 μM	
Piperleine B (**14**)	0 ± 3	21 ± 4	64 ± 5[b]	73 ± 8[b]	92 ± 3[b]	2.9

Each value represents the mean ± SEM (N = 4).
Significantly different from the control.
[a] p <0.05.
[b] p <0.01.
[c] Cytotoxic effect was observed.
[d] Commercial silybin was purchased from Funakoshi Co, Ltd. (Tokyo, Japan).

Table 6
Inhibitory effects of the chemical constituents from the fruit of *P. chaba* on D-GalN/TNF-α-induced cytotoxicity in primary cultured mouse hepatocytes

	Inhibition (%)					IC_{50} (μM)
	0 μM	1 μM	3 μM	10 μM	30 μM	
Piperchabamide E (**1**)	0 ± 2		34 ± 2[b]	71 ± 2[b]	98 ± 6[b]	4.9
Piperchabamide G (**2**)	0 ± 1		43 ± 6[b]	104 ± 4[b]		ca. 4
Piperchabamide H (**3**)	0 ± 5		24 ± 3[b]	42 ± 3[b]	118 ± 4[b]	ca. 11
Piperonal (**4**)	0 ± 4		20 ± 1[b]	23 ± 5[b]	39 ± 2[b]	
Methyl piperate (**5**)	0 ± 2		18 ± 3	29 ± 2[b]	21 ± 5	
Piperchabamide A (**6**)	0 ± 4		29 ± 1[b]	32 ± 2[b]	70 ± 7[b]	14
$\Delta^{\alpha,\beta}$-Dihydropiperine (= piperanine, **9**)	0 ± 2		31 ± 5[a]	50 ± 2[b]	53 ± 2[b]	17
Piperine (**10**)	0 ± 3		23 ± 5[b]	46 ± 4[b]	68 ± 3[b]	12
Piperoleine B (**14**)	0 ± 10	20 ± 3[a]	29 ± 3[b]	33 ± 1[b]	64 ± 7[b]	17
Pipernonaline (**15**)	0 ± 4		17 ± 6	31 ± 3[b]	48 ± 2[b]	> 30
Piperchabamide B (**16**)	0 ± 2		63 ± 6[b]	74 ± 3[b]	78 ± 5[b]	<3
Piperundecalidine (**18**)	0 ± 2	27 ± 6[a]	40 ± 3[b]	49 ± 8[b]	58 ± 5[b]	11
Piperchabamide C (**19**)	0 ± 3		42 ± 5[b]	52 ± 1[b]	68 ± 5[b]	6.7
5,6-Dihydropiperlonguminine (**21**)	0 ± 6	34 ± 12	43 ± 9[a]	43 ± 5[b]	66 ± 6[b]	8.2
Piperlonguminine (**22**)	0 ± 6	27 ± 4	50 ± 6[b]	54 ± 6[b]	66 ± 3[b]	ca. 3
Retrofractamide C (**23**)	0 ± 3	30 ± 13	51 ± 2[b]	31 ± 6[b]	37 ± 2[b]	ca. 3
Piperchabamide D (**24**)	0 ± 1		57 ± 3[b]	77 ± 3[b]	37 ± 4[b]	<3
Retrofractamide A (**26**)	0 ± 3		32 ± 5[b]	35 ± 5[b]	60 ± 5[b]	21
Pipercide (= retrofractamide B, **27**)	0 ± 3	32 ± 5	54 ± 4[b]	51 ± 3[b]	26 ± 2[b,c]	2.6
Guineensine (**28**)	0 ± 2		26 ± 2[b]	33 ± 3[b]	12 ± 1[b,c]	
Brachystamide B (**29**)	0 ± 3		22 ± 3[b]	11 ± 2[a]	-6 ± 2	
N-Isobutyl-(2*E*,4*E*)-deca-2,4-dienamide (**30**)	0 ± 7		19 ± 3[a]	25 ± 3[b]	42 ± 3[b]	
N-Isobutyl-(2*E*,4*E*)-dodeca-2,4-dienamide (**31**)	0 ± 3	12 ± 2	21 ± 3[b]	31 ± 6[b]	44 ± 4[b]	
N-Isobutyl-(2*E*,4*E*)-octadeca-2,4-dienamide (**34**)	0 ± 5	15 ± 10	37 ± 2[b]	41 ± 1[b]	43 ± 5[b]	
N-Isobutyl-(2*E*,4*E*14*Z*)-eicosa-2,4,14-trienamide (**37**)	0 ± 4	10 ± 5	3 ± 2	19 ± 12	27 ± 6	
Silybin[d]	0 ± 2	11 ± 9	19 ± 6	37 ± 5[b]	93 ± 4[b]	15

Each value represents the mean ± SEM ($N = 4$).
Significantly different from the control.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] Cytotoxic effect was observed.
[d] Commercial silybin was purchased from Funakoshi Co., Ltd (Tokyo, Japan).

silica gel RP-18 WF_{254S} (Merck, 0.25 mm). Detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4, followed by heating.

3.2. Plant material

This item was described in a previous report.[2]

Table 7
Inhibitory effects of the aqueous acetone extract from the fruit of *P. chaba* and its chemical constituents on NO production in LPS-activated mouse peritoneal macrophages

	Inhibition (%)					IC_{50} (μg/mL)
	0 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	
80% Aqueous acetone ext.	0 ± 2	4 ± 1	10 ± 3[b]	34 ± 2[b]	90 ± 1[b]	44
	0 μM	3 μM	10 μM	30 μM	100 μM	IC_{50} (μM)
Piperchabamide E (**1**)	0 ± 4	1 ± 4	6 ± 1	16 ± 1[b]	46 ± 2[b]	
Piperchabamide G (**2**)	0 ± 2	−2 ± 2	10 ± 3	75 ± 1[b]	81 ± 1[b]	28
Piperchabamide H (**3**)	0 ± 2	−2 ± 1	3 ± 2	16 ± 2[b]	21 ± 1[b]	
Piperonal (**4**)	0 ± 2	1 ± 1	2 ± 1	2 ± 4	7 ± 1	
Methyl piperate (**5**)	0 ± 3	2 ± 3	5 ± 2	8 ± 1	14 ± 2[b]	
Piperchabamide A (**6**)	0 ± 2	3 ± 1	27 ± 1[b]	98 ± 1[b]	100 ± 1[b,c]	20
$\Delta^{\alpha,\beta}$-Dihydropiperine (= piperanine, **9**)	0 ± 1	2 ± 1	3 ± 3	7 ± 2	20 ± 1[b]	
Piperine (**10**)	0 ± 2	2 ± 2	2 ± 3	11 ± 5	49 ± 4[b]	
Pipernonaline (**15**)	0 ± 3	-3 ± 2	7 ± 1	47 ± 1[b]	99 ± 1[b,c]	
Piperchabamide B (**16**)	0 ± 5	5 ± 1	11 ± 3[a]	24 ± 1[b]	99 ± 1[b,c]	
Dehydropipernonaline (**17**)	0 ± 2	23 ± 4[b]	25 ± 5[b]	28 ± 7[b]	101 ± 1[b,c]	
Piperundecalidine (**18**)	0 ± 8	6 ± 10	3 ± 6	46 ± 3[b]	97 ± 1[b,c]	
Piperchabamide C (**19**)	0 ± 4	9 ± 2	10 ± 2[a]	23 ± 1[b]	86 ± 1[b]	42
5,6-Dihydropiperlonguminine (**21**)	0 ± 9	−13 ± 8	−1 ± 4	−4 ± 5	25 ± 3[a]	
Piperchabamide D (**24**)	0 ± 4	−1 ± 2	7 ± 1	10 ± 2[a]	22 ± 1[b]	
Retrofractamide A (**26**)	0 ± 5	4 ± 4	−12 ± 3	−8 ± 4	−14 ± 7	
L-NMMA[60,61]	0 ± 3	1 ± 3	20 ± 3[b]	43 ± 2[b]	71 ± 2[b]	36
CAPE[60,61]	0 ± 2	6 ± 5	44 ± 3[b]	86 ± 1[b]	100 ± 1[b,c]	11

Each value represents the mean ± SEM ($N = 4$).
Significantly different from the control.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] Cytotoxic effect was observed.

Table 8
Inhibitory effects of the aqueous acetone extract from the fruit of *P. chaba* and its chemical constituents on TNF-α-induced cytotoxicity in L929 cells

	Inhibition (%)					IC_{50} (μg/mL)
	0 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	
80% Aqueous acetone ext.	0 ± 1	8 ± 3	19 ± 1[b]	85 ± 2[b]		14
	0 μM	3 μM	10 μM	30 μM	100 μM	IC_{50} (μM)
Piperchabamide E (**1**)	0 ± 2	0 ± 5	−4 ± 2	−1 ± 3	22 ± 3[b]	
Piperchabamide G (**2**)	0 ± 3	8 ± 2	12 ± 4	20 ± 6[a]	18 ± 6[a]	
Piperchabamide H (**3**)	0 ± 2	3 ± 3	−2 ± 1	−4 ± 1	5 ± 3	
Piperonal (**4**)	0 ± 4	1 ± 8	−4 ± 6	0 ± 8	10 ± 7	
Methyl piperate (**5**)	0 ± 1	9 ± 2	10 ± 2	13 ± 2[b]	15 ± 3[b]	
Piperchabamide A (**6**)	0 ± 4	23 ± 5[b]	35 ± 3[b]	71 ± 1[b]	90 ± 5[b]	13
$\Delta^{\alpha,\beta}$-Dihydropiperine (= piperanine, **9**)	0 ± 1	12 ± 4	11 ± 2	2 ± 1	−1 ± 2	
Piperine (**10**)	0 ± 2	10 ± 3	17 ± 5[b]	22 ± 1[b]	82 ± 2[b]	42
Piperoleine B (**14**)	0 ± 2	7 ± 3	18 ± 1[b]	25 ± 1[b]	66 ± 4[b]	64
Pipernonaline (**15**)	0 ± 3	8 ± 1	10 ± 3	−5 ± 1	−10 ± 2	
Piperchabamide B (**16**)	0 ± 4	4 ± 7	12 ± 3	19 ± 3[a]	94 ± 4[b]	33
Piperundecalidine (**18**)	0 ± 1	5 ± 3	−1 ± 9	24 ± 1[b]	29 ± 3[b]	
Piperchabamide C (**19**)	0 ± 4	−2 ± 3	2 ± 7	25 ± 4[b]	92 ± 5[b]	42
Piperlonguminine (**22**)	0 ± 1	16 ± 2	25 ± 2[b]	25 ± 1[b]	23 ± 2[b]	
Piperchabamide D (**24**)	0 ± 4	8 ± 4	−3 ± 3	−23 ± 2	−20 ± 3	
Pipercide (= retrofractamide B, **27**)	0 ± 4	1 ± 3	5 ± 2	1 ± 2	0 ± 3	
Guineensine (**28**)	0 ± 2	9 ± 1	2 ± 1	−9 ± 7	−18 ± 2	
Brachystamide B (**29**)	0 ± 1	4 ± 1	1 ± 1	−11 ± 1	−15 ± 1	
N-Isobutyl-(2*E*,4*E*)-deca-2,4-dienamide (**30**)	0 ± 3	4 ± 1	10 ± 1[a]	18 ± 2[b]	19 ± 3[b]	
N-Isobutyl-(2*E*,4*E*)-dodeca-2,4-dienamide (**31**)	0 ± 5	0 ± 5	−2 ± 5	−3 ± 4	34 ± 6[b]	
N-Isobutyl-(2*E*,4*E*)-octadeca-2,4-dienamide (**34**)	0 ± 2	5 ± 2	−2 ± 1	−4 ± 3	−3 ± 2	
N-Isobutyl-(2*E*,4*E*14*Z*)-eicosa-2,4,14-trienamide (**37**)	0 ± 3	14 ± 1[b]	32 ± 1[b]	45 ± 2[b]	82 ± 2[b]	25
Curcumin[14–16]	0 ± 2	25 ± 2[b]	47 ± 4[b]	52 ± 4[b]		20

Each value represents the mean ± SEM ($N = 4$).
Significantly different from the control.
[a] $p<0.05$.
[b] $p<0.01$.

Table 9
Inhibitory effects of piperine (**10**) on D-GalN/LPS-induced liver injury in mice

Treatment	Dose (mg/kg, po)	*n*	sAST		sALT	
			(Karmen unit)	Inhibition (%)	(Karmen unit)	Inhibition (%)
Normal (vehicle)	—	5	95 ± 5[b]	—	19 ± 1[b]	—
Control (D-GalN/LPS)	—	8	9126 ± 1477	—	9830 ± 1605	—
Piperine (**10**)	2.5	4	9098 ± 1195	0.3	7555 ± 796	23.2
	5	7	4545 ± 1259[a]	50.7	4817 ± 1489[a]	51.1
	10	8	450 ± 145[b]	96.1	575 ± 196[b]	94.3
Hydrocortisone	10	7	627 ± 262[b]	94.2	247 ± 123[b]	97.7

Each value represents the mean ± SEM.
Significantly different from the control.
[a] $p<0.05$.
[b] $p<0.01$.

Table 10
Inhibitory effects of piperine (**10**) on the increase in serum TNF-α levels induced by D-GalN/LPS in mice

	Dose (mg/kg, po)	*n*	TNF-α (pg/mL)	Inhibition (%)
Normal (vehicle)	—	4	4 ± 3[a]	—
Control (D-GalN/LPS)	—	6	233 ± 46	—
Piperine (**10**)	5	4	197 ± 52	15.7
	10	4	212 ± 27	9.2
	20	4	238 ± 71	−2.2
Hydrocortisone	10	4	22 ± 2[a]	92.1

Each value represents the mean ± SEM.
Significantly different from the control.
[a] $p<0.01$.

3.3. Extraction and isolation

The dried fruit of *P. chaba* (4.0 kg) was finely cut and extracted four times with 80% (v/v) aqueous acetone at room temperature for one day. Evaporation of the solvent under reduced pressure provided an 80% aqueous acetone extract (788 g, 19.7% from the dried fruit). The 80% aqueous acetone extract (463 g) was partitioned between EtOAc–H_2O (1:1, v/v) and removal of the solvent under reduced pressure yielded an EtOAc-soluble fraction (227 g, 9.7%) and an aqueous phase. The EtOAc-soluble fraction (118 g) was subjected to normal-phase silica gel CC [3.0 kg, *n*-hexane–EtOAc (15:1→10:1→5:1→2:1→1:1, v/v)→MeOH] to give nine fractions [Fr. 1 (7.3 g), 2 (4.0 g), 3 (3.1 g), 4 (22.0 g), 5 (3.8 g), 6 (1.8 g), 7 (11.1 g), 8 (44.2 g), and 9 (20.7 g)] as described previously.[2]

The fraction 2 (3.2 g) was separated by reversed-phase silica gel CC [120 g, MeOH–H_2O (70:30→90:10, v/v)→MeOH] to give piperonal (**4**, 45.9 mg, 0.0047%) together with methyl piperate (**5**, 1.0 g, 0.11%). The fraction 3 (3.1 g) was separated by reversed-phase silica gel CC [630 g, MeOH–H_2O (60:40→70:30→80:20→90:10→95:5, v/v)→MeOH] and finally HPLC [CH_3CN–H_2O (35:65, v/v)] to give 3,4-methylenedioxycinnamaldehyde (23 mg, 0.0019%). The fraction 4 (20.0 g) was separated by reversed-phase silica gel CC [700 g, MeOH–H_2O (60:40→70:30→80:20→90:10→95:5, v/v)→MeOH] to give eight fractions [Fr. 4-1 (1.2 g), 4-2 (0.21 g), 4-3 (0.29 g), 4-4 (1.1 g), 4-5 (7.3 g), 4-6 (4.6 g), 4-7 (3.9 g), and 4-8 (1.4 g)]. The fraction 4-1 (1.2 g) was purified by HPLC [CH_3CN–H_2O (35:65, v/v)] to give benzenepropanoic acid (980 mg, 0.090%). The fraction 4-2 (0.21 g) was subjected to preparative HPLC [CH_3CN–H_2O (50:50, v/v)] to give piperchabamide A (**6**, 27 mg, 0.0029%) and *N*-isobutyl-(2*E*,4*E*)-deca-2,4-dienamide (**30**, 27 mg, 0.0025%). The fraction 4-3 (0.29 g) was subjected to preparative HPLC [CH_3CN–H_2O (65:35, v/v)] to give **30** (80 mg, 0.0074%), *N*-isobutyl-(2*E*,4*E*)-dodeca-2,4-dienamide (**31**, 37 mg, 0.0034%), and *N*-isobutyl-(2*E*,4*E*,8*Z*)-tetradeca-2,4,8-trienamide (**35**, 18 mg, 0.0017%). The fraction 4-4 (1.1 g) was purified by HPLC [CH_3CN–H_2O (83:17, v/v)] to give *N*-isobutyl-(2*E*,4*E*)-tetradeca-2,4-dienamide (**32**, 49 mg, 0.0044%). The fraction 4-5 (0.91 g) was purified by HPLC [MeOH–H_2O (90:10, v/v)] to give *N*-isobutyl-(2*E*,4*E*)-hexadeca-2,4-dienamide (**33**, 15 mg, 0.011%). The fraction 4-6 (0.80 g) was purified by HPLC [MeOH–H_2O (90:10, v/v)] to give *N*-isobutyl-(2*E*,4*E*)-octadeca-2,4-dienamide (**34**, 185 mg, 0.18%), *N*-isobutyl-(2*E*,4*E*,12*Z*)-octadeca-2,4,12-trienamide (**36**, 12 mg, 0.0057%), and *N*-isobutyl-(2*E*,4*E*,14*Z*)-eicosa-2,4,14-trienamide (**37**, 362 mg, 0.18%). The fraction 4-7 (0.91 g) was purified by HPLC [MeOH–H_2O (90:10, v/v)] to give **34** (127 mg, 0.048%). The fraction 5 (3.8 g) was subjected to reversed-phase silica gel CC [200 g, MeOH–H_2O (60:40→70:30→80:20→90:10, v/v)→MeOH] to furnish four fractions [Fr. 5-1 (1.2 g), 5-2 (0.62 g), 5-3 (1.3 g), and 5-4 (0.68 g)]. The fraction 5-1 (1.2 g) was subjected to HPLC [CH_3CN–H_2O (35:65, v/v)] to give benzenepropanoic acid (489 mg, 0.040%) and **30** (94 mg, 0.0085%). The fraction 5-2 (0.62 g) was subjected to HPLC [CH_3CN–H_2O (70:30, v/v)] to give piperchabamide D (**24**, 20 mg, 0.0016%), pipercide [= retrofractamide B (**27**, 100 mg, 0.0083%), and guineensine (**28**, 292 mg, 0.024%). The fraction 5-3 (1.3 g) was further purified by HPLC [CH_3CN–H_2O (75:25, v/v)] to give dihydroguineensine (**25**, 18 mg, 0.0015%), **28** (690 mg, 0.057%), and brachystamide B (**29**, 83 mg, 0.0069%). The fraction 6 (1.6 g) was subjected to reversed-phase silica gel CC [200 g, MeOH–H_2O (60:40→70:30→80:20→90:10→95:5, v/v)→MeOH] to give five fractions [Fr. 6-1 (0.14 g), 6-2 (0.07 g), 6-3 (0.80 g), 6-4 (0.39 g), and 6-5 (0.20 g)]. The fraction 6-1 (0.14 g) was further purified by HPLC [CH_3CN–H_2O (45:55, v/v)] to give *N*-cinnamoylpiperidine (**7**, 17 mg, 0.0016%). The fraction 6-3 (0.80 g) was subjected to HPLC [CH_3CN–H_2O (67:33, v/v)] to give piperoleine B (**14**, 47 mg, 0.0042%), dehydropipernonaline (**17**, 97 mg, 0.0088%), retrofractamide C (**23**, 103 mg, 0.0093%), **24** (12 mg, 0.0011%), retrofractamide A (**26**, 36 mg, 0.0032%), and **27** (322 mg, 0.029%). The fraction 6-4 (0.39 g) was further purified by HPLC [CH_3CN–H_2O (67:33, v/v)] to give **14** (3 mg, 0.0002%), piperchabamide B (**16**, 19 mg, 0.0018%), piperundecalidine (**18**, 30 mg, 0.0028%), piperchabamide C (**19**, 34 mg, 0.0032%), **24** (11 mg, 0.0010%), **27** (94 mg, 0.0089%), and **28** (58 mg, 0.0055%). The fraction 7 (5.9 g) was subjected to reversed-phase silica gel CC [270 g, MeOH–H_2O (60:40→70:30→80:20→90:10, v/v)→MeOH] to yield 11 fractions [Fr. 7-1 (0.17 g), 7-2 (0.28 g), 7-3 (0.27 g), 7-4 (1.0 g), 7-5 (0.09 g), 7-6 (0.58 g), 7-7 (2.7 g), 7-8 (0.30 g), 7-9 (0.09 g), 7-10 (0.17 g), and 7-11 (0.25 g)]. The fraction 7-2 (0.28 g) was further purified by HPLC [CH_3CN–H_2O (45:55, v/v)] to give 5,6-dihydropiperlonguminine (**21**, 84 mg, 0.015%). The fraction 7-4 (1.0 g) was subjected to HPLC [CH_3CN–H_2O (45:55, v/v)] to furnish piperchabamide E (**1**, 27 mg, 0.0046%), piperine (**10**, 43 mg, 0.0075%), isopiperine (**11**, 157 mg, 0.027%), isochavicine (**12**, 111 mg, 0.020%), chavicine (**13**, 24 mg, 0.0042%), piperlonguminine (**22**, 76 mg, 0.013%), and piperic acid isopentyl amide (**38**, 7 mg, 0.0011%). The fraction 7-6 (0.58 g) was purified by HPLC [CH_3CN–H_2O (67:33, v/v)] to give pipernonaline (**15**, 36 mg, 0.0063%), **17** (145 mg, 0.0025%), **23** (107 mg, 0.019%), and **26** (25 mg, 0.0043%). The fraction 7-7 (1.0 g) was further purified by HPLC [CH_3CN–H_2O (67:33, v/v)] to give **15** (874 mg, 0.40%), **17** (64 mg, 0.029%), and **23** (18 mg, 0.0081%). The fraction 7-8 (0.30 g) was subjected to HPLC [CH_3CN–H_2O (67:33, v/v)] to furnish **14** (8 mg, 0.0016%), **15** (198 mg, 0.039%), **17** (7 mg, 0.0014%), and **27** (14 mg, 0.0027%). The fraction 7-10 (0.17 g) was separated by HPLC [CH_3CN–H_2O (70:30, v/v)] to give piperchabamide H (**3**, 12 mg, 0.0020%), **16** (13 mg, 0.0023%), and **18** (79 mg, 0.014%). The fraction 8 (44.2 g) was recrystallized in MeOH to give **10** (30.8 g, 2.64%). The mother liquor fraction (13.4 g) was subjected to reverse-phase silica gel CC [450 g, MeOH–H_2O (60:40→90:10, v/v)→MeOH] to give nine fractions [Fr. 8-1 (0.29 g), 8-2 (0.32 g), 8-3 (6.6 g), 8-4 (4.8 g), 8-5 (0.39 g), 8-6 (0.74 g), 8-7 (0.17 g), 8-8 (0.32 g), and 8-9 (0.17 g)]. The fraction 8-2 (0.32 g) was separated by HPLC [CH_3CN–H_2O (50:50, v/v)] to give fragaramide (**20**, 7 mg, 0.0006%). The fraction 8-3 (2.0 g) was separated by normal-phase silica gel CC [500 g, *n*-hexane–EtOAc (4:1→3:1→2.5:1→1:1, v/v)→MeOH] and finally HPLC [CH_3CN–H_2O (55:45, v/v)] to give ilepcimide (**8**, 16 mg, 0.0048%), $\Delta^{\alpha,\beta}$-dihydropiperine [= piperanine (**9**, 1268 mg, 0.36%)], and **22** (554 mg, 0.15%). The fraction 8-4 (0.88 g) was further purified by HPLC [CH_3CN–H_2O (45:55, v/v)] to give **9** (126 mg, 0.057%), **10** (483 mg, 0.22%), **11** (24 mg, 0.011%), **12** (32 mg, 0.015%), and **22** (130 mg, 0.059%). The fraction 8-5 (0.32 g) was subjected to HPLC [CH_3CN–H_2O (45:55, v/v)] to give **10** (124 mg, 0.013%), **11** (18 mg, 0.0018%), and **12** (39 mg, 0.0041%). The fraction 8-6 (0.74 g) was further purified by HPLC [CH_3CN–H_2O (60:40, v/v)] to give piperchabamide G (**2**, 76 mg, 0.0064%), **15** (266 mg, 0.022%), and **17** (165 mg, 0.014%). The fraction 8-7 (0.17 g) was separated by HPLC [CH_3CN–H_2O (70:30, v/v)] to give **3** (51 mg, 0.0042%) and **15** (27 mg, 0.0023%). The fraction 8-8 (0.32 g) was further purified by HPLC [CH_3CN–H_2O (60:40, v/v)] to give **3** (29 mg, 0.0023%).

3.3.1. Piperchabamide E (1)

Colorless oil, $[\alpha]_D^{24}$ +18.7 (*c* 0.72, $CHCl_3$). High-resolution EIMS: Calcd for $C_{17}H_{21}NO_3$ $(M)^+$: 287.1521. Found: 287.1523. UV [EtOH, nm (log ε)]: 241 (4.2), 252 (4.1), 308 (4.2), 338 (4.3). IR (film): 2963, 1646, 1545, 1506, 1489, 1446, 1257, 1238, 1039, 991 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: given in Table 3. ^{13}C NMR (125 MHz, $CDCl_3$) δ_C: given in Table 4. EIMS *m/z*: 287 (M^+, 48), 201 (100).

3.3.2. Piperchabamide G (2)

An amorphous powder, $[\alpha]_D^{28}$ 0 (*c* 0.63, $CHCl_3$). High-resolution EIMS: Calcd for $C_{34}H_{40}N_2O_6$ $(M)^+$: 572.2886. Found: 572.2884. UV [EtOH, nm (log ε)]: 235 (3.9), 288 (3.8). IR (film): 2936, 1624, 1485, 1441, 1244, 1040, 928 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: given in Table 3. ^{13}C NMR (125 MHz, $CDCl_3$) δ_C: given in Table 4. EIMS *m/z*: 572 (M^+, 48), 112 (100).

3.3.3. Piperchabamide H (3)

An amorphous powder, $[\alpha]_D^{27}$ 0 (*c* 0.38, $CHCl_3$). High-resolution EIMS: Calcd for $C_{38}H_{46}N_2O_6$ $(M)^+$: 626.3355. Found: 626.3352. UV [EtOH, nm (log ε)]: 362 (4.2), 288 (4.0). IR (film): 2932, 1628, 1487, 1443, 1248, 1040, 930 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: given in Table 3. ^{13}C NMR (125 MHz, $CDCl_3$) δ_C: given in Table 4. EIMS *m/z*: 626 (M^+, 48), 112 (100).

3.4. Acid hydrolysis of 1

A solution of **1** (10 mg) in 6 M HCl (1.0 mL) was heated at 110 °C overnight in a sealed tube. After cooling, the reaction mixture was basified (pH 10) with aq NaOH and then the mixture was extracted with *n*-hexane. The *n*-hexane phase was evaporated in vacuo to give a residue, which was subjected to HPLC analysis [column: Cosmosil $5C_{18}$-MS-II, 250 × 4.6 mm i.d.; mobile phase: MeOH–H_2O (50:50, v/v); detection: RI and OR; flow rate: 1.0 mL/min] to identify (*R*)-2-methylbutylamine [t_R 9.1 min (positive)].

3.5. Bioassay

3.5.1. Reagents

LPS (from *Salmonella enteritidis*), minimum essential medium (MEM), and William's E medium were purchased from Sigma Chemical (St. Louis, MO, USA); fetal calf serum (FCS) was from Life Technologies (Rockville, MD, USA); and other chemicals were from Wako Pure Chemical Industries (Osaka, Japan). 96-Well microplates were purchased from Nalge Nunc International (Naperville, IL, USA).

3.5.2. Animals

Male ddY mice weighing about 25–30 g were purchased from Kiwa Laboratory Animal Co., Ltd, (Wakayama, Japan). The animals were housed at a constant temperature of 23 ± 2 °C and were fed standard laboratory chow (MF, Oriental Yeast Co., Ltd, Tokyo, Japan). The animals were fasted for 20–24 h prior to the beginning of the experiment, but were allowed free access to tap water. All of the experiments were performed with conscious mice unless otherwise noted. The experimental protocol was approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

3.5.3. Protective effects on D-GalN/LPS-induced liver injury in mice

The method described by Tiegs et al.[65] was modified and used for this experiment. Briefly, male ddY mice weighing about 25–30 g were fasted for 20 h before the experiment. D-GalN (350 mg/kg) and LPS (10 μg/kg) dissolved in saline were injected intraperitoneally to produce liver injury. Each test sample was given orally 1 h before D-GalN/LPS was injected. Blood samples were collected from the infraorbital venous plexus 10 h after D-GalN/LPS injection. sAST and sALT levels were determined using the Reitman-Frankel method (commercial kit, S.TA-Test Wako, Wako Pure Chemical Industries Co. Ltd). Hydrocortisone was used as a reference compound. Test samples were suspended with 5% Arabic gum solution, and the solution was administered orally at 10 mL/kg in each experiment, while the vehicle was given orally at 10 mL/kg in the corresponding control group.

3.5.4. Protective effects on cytotoxicity induced by D-GalN or D-GalN/TNF-α in primary cultured mouse hepatocytes

The hepatoprotective effects of the constituents were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay of primary cultured mouse hepatocytes.[23–31] Hepatocytes were isolated from male ddY mice (30–35 g) by collagenase perfusion method. A cell suspension of 4×10^4 cells in 100 μL William's E medium containing FCS (10%), penicillin G (100 units/mL), and streptomycin (100 μg/mL) was inoculated in a 96-well microplate and precultured for 4 h at 37 °C under a 5% CO_2 atmosphere. The fresh medium (100 μL) containing D-GalN (2 mM) with or without the test sample was added, and the hepatocytes were cultured for 44 h for the D-GalN-induced cytotoxicity test. For the D-GalN/TNF-α-induced cytotoxicity test, the fresh medium (100 μL) containing D-GalN (2 mM) and TNF-α (40 ng/mL) with or without the test sample was added, and the hepatocytes were cultured for 20 h. The medium was then exchanged with 100 μL of fresh medium, and 10 μL of MTT [5 mg/mL in phosphate buffered saline (PBS)] solution was added to the medium. After 4 h of culture, the medium was removed, and 100 μL of isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced in the cells. The optical density (OD) of the formazan solution was measured with a microplate reader at 570 nm (reference: 655 nm). Inhibition (%) was obtained using following formula.

$$\text{Inhibition } (\%) = [(\text{OD(sample)} - \text{OD(control)})/(\text{OD(normal)} - \text{OD(control)})] \times 100$$

3.5.5. Effects on production of NO in LPS-stimulated mouse peritoneal macrophages

The screening test for NO production using TGC-induced mouse peritoneal macrophages was performed as described previously.[60,61] Briefly, peritoneal exudate cells (5×10^5 cells/well) were collected from the peritoneal cavities of male ddY mice, suspended in 100 μL of RPMI 1640 supplemented with 5% FCS, penicillin (100 units/mL), and streptomycin (100 μg/mL), and pre-cultured in 96-well microplates at 37 °C in 5% CO_2 in air for 1 h. Nonadherent cells were removed by washing with PBS, and the adherent cells were cultured in 200 μL of fresh medium containing 10 μg/mL LPS and various concentrations of test compound for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite (NO_2^-) in the culture medium using Griess reagent. Cytotoxicity was determined with the MTT colorimetric assay, after 20 h incubation with test compounds. Each test compound was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration 0.5%). N^G-Monomethyl-L-arginine (L-NMMA, an NOS inhibitor) and caffeic acid phenethyl ester (CAPE, an inhibitor of NF-κB activation) were used as reference compounds. Inhibition (%) was calculated using the following formula, and the IC_{50} value was determined graphically ($N = 4$).

$$\text{Inhibition } (\%) = [(A - B)/(A - C)] \times 100$$

$A - C$: NO_2^- concentration (μg/mL or μM)

[A : LPS (+), sample (−); B : LPS (+), sample (+); C : LPS (−), sample (−)]

3.5.6. Inhibitory effects on TNF-α-induced cell death in L929 cells

L929 cells (Dainippon Pharmaceuticals, Osaka, Japan) were maintained in Eagle's MEM (Sigma–Aldrich) containing 10% FCS, 1% MEM Non-Essential Amino Acids (Invitrogen), penicillin G (100 units/mL), and streptomycin (100 μg/mL) at 37 °C under a 5% CO_2 atmosphere. The cells were inoculated in a 96-well tissue culture plate [3×10^4 cells/well in 100 μL/well in MEM]. After 20 h of incubation in the medium containing actinomycin D (0.5 μg/mL) and TNF-α (20 pg/mL) with or without the test sample, the viability of the cells was assessed using the MTT colorimetric assay (vide ante). Each test compound was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration 0.5%).

3.5.7. Effects on increase in serum TNF-α levels induced by D-GalN/LPS in mice

Male ddY mice weighing about 25–30 g were fasted for 20 h before the experiment. D-GalN (350 mg/kg) and LPS (10 μg/kg) dissolved in saline were injected intraperitoneally to produce liver injury. Each test sample was given orally 1 h before D-GalN/LPS was injected. Blood samples were collected from the infraorbital ve-

nous plexus 1.5 h after D-GalN/LPS injection. The serum TNF-α level was determined using an ELISA kit (GE Healthcare Sciences).[66]

3.6. Statistics

Values are expressed as means ± SEM. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical analysis. Probability (p) values of less than 0.05 were considered to represent a statistically significant difference.

Acknowledgements

M.Y. and H.M. were supported by the 21st COE Program, Academic Frontier Project, and a Grant-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. T.M. and K.N. were supported by a Grant-in Aid for Scientific Research from the 'High-tech Research Center' Project for Private Universities: matching fund subsidy from a Grant-in Aid for Scientific Research from MEXT, 2007–2011. T.M. was also supported by Takeda Science Foundation.

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