Medicinal Foodstuffs. XXXIV.[1] Structures of New Prenylchalcones and Prenylflavanones with TNF-α and Aminopeptidase N Inhibitory Activities from *Boesenbergia rotunda*

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The methanolic extract from the rhizomes of *Boesenbergia rotunda* (Zingiberaceae) was found to show inhibitory effect on tumor necrosis factor-α (TNF-α)-induced cytotoxicity in L929 cells (IC_{50}=6.1 μg/ml). By bioassay-guided separation, four new prenylcalcones, (+)-krachaizin A (1a), (−)-krachaizin A (1b), (+)-krachaizin B (2a), and (−)-krachaizin B (2b), and four new prenylflavanones, rotundaflavones Ia (3a), Ib (3b), IIa (4a), and IIb (4b), were isolated together with 18 known constituents (5a—7b and 8—19). The structures of eight new compounds were elucidated on the basis of physicochemical evidence. Among them, (+)-krachaizin B (2a), (−)-krachaizin B (2b), (+)-4-hydroxypanduratin A (6a), (−)-4-hydroxypanduratin A (6b), (+)-isopanduratin A (7a), (−)-isopanduratin A (7b), alpinetin (10), cardamonin (14), and 2,6-dihydroxy-4-methoxydihydrochalcone (15) significantly inhibited TNF-α-induced cytotoxicity in L929 cells at 10 μM. In addition, 2a, 2b, (+)-panduratins A (5a), (−)-panduratin A (5b), 6a, 7b, and geranyl-2,4-dihydroxy-6-phenylbenzoate (17) were found to show strong inhibitory effects on aminopeptidase N activity.

Key words *Boesenbergia rotunda*; Zingiberaceae; krachaizin; rotundaflavone; tumor necrosis factor-α inhibitory activity; aminopeptidase N inhibitor

Boesenbergia rotunda (LINN.) MANSF. [*syn. B. pandulata* (ROXB.) SCHLTR.] (Zingiberaceae), is distributed in Southeastern Asian countries such as Myanmar, Indonesia, Malaysia, and Thailand (Thai Ginseng in English, Krachai in Thai).[1—3] The rhizomes of *B. rotunda* also have been used as a spice in Thailand and have been used for the treatment of oral diseases (dry mouth), stomach discomfort, stomach pain, leucorrhea, diuretic, dysentery, and inflammation, *etc.*[1—3] We previously reported that six optically active Diels–Alder type addition prenylcalcones, (+)-panduratin A (**5a**), (−)-panduratin A (**5b**), (+)-4-hydroxypanduratin A (**6a**), (−)-4-hydroxypanduratin A (**6b**), (+)-isopanduratin A (**7a**), and (−)-isopanduratin A (**7b**), were isolated from the rhizomes of this herbal medicine together with 12 known constituents (**8**—**19**).[1] In addition, the methanolic extract and principal constituents, the enantiomeric mixtures of panduratin A (**5a**, **b**) and 4-hydroxypanduratin A (**6a**, **b**), and pinocembrin (**9**), were found to show gastroprotective effects on ethanol- or indomethacin-induced gastric mucosal lesions in rats.[1] During the course of our characterization studies on Thai medicinal foods such as *Albizia myriophylla*,[4] *Salacia chinensis*,[5—7] *Alpinia galanga*,[8—12] *Piper chaba*,[13,14] *Curcuma zedoaria* (Thai Zedoary),[15] *Erycibe expansa*,[16—18] and *Borassus flabellifer*,[19] the methanolic extract from the dried rhizomes of *B. rotunda* was found to inhibit on tumor necrosis factor-α (TNF-α)-induced cytotoxicity in L929 cells. By bioassay-guided separation, four new prenylcalcones, (+)-krachaizin A (**1a**), (−)-krachaizin A (**1b**), (+)-krachaizin B (**2a**), and (−)-krachaizin B (**2b**), and four new prenylflavanones, rotundaflavones Ia (**3a**), Ib (**3b**), IIa (**4a**), and IIb (**4b**), were then isolated. This paper deals with the isolation and structure elucidation of eight new compounds (**1a**—**4b**) as well as TNF-α and aminopeptidase N inhibitory activities of the isolated compounds.

The dried rhizomes of *B. rotunda*, which were cultivated in Nakhonsithammarat province of Thailand, were extracted with methanol to give the methanolic extract (10.2% from the dried rhizomes) as reported previously.[1] The methanolic extract was subjected to normal- and reversed-phase silica gel column chromatographies, and finally HPLC to give (+)-krachaizin A (**1a**, 0.0015%), (−)-krachaizin A (**1b**, 0.0014%), (+)-krachaizin B (**2a**, 0.0022%), (−)-krachaizin B (**2b**, 0.0018%), rotundaflavones Ia (**3a**, 0.0010%), Ib (**3b**, 0.0025%), IIa (**4a**, 0.00048%), and IIb (**4b**, 0.00037%) together with 18 known constituents (**5a**—**7b** and **8**—**19**).[1]

Structures of (+)-Krachaizin A (1a), (−)-Krachaizin A (1b), (+)-Krachaizin B (2a), and (−)-Krachaizin B (2b) (+)-Krachaizin A (**1a**) and (−)-krachaizin A (**1b**) were isolated as white powders with positive and negative optical rotation (**1a**: $[\alpha]_D^{22}$ +82°, **1b**: $[\alpha]_D^{23}$ −78° both in MeOH), re-

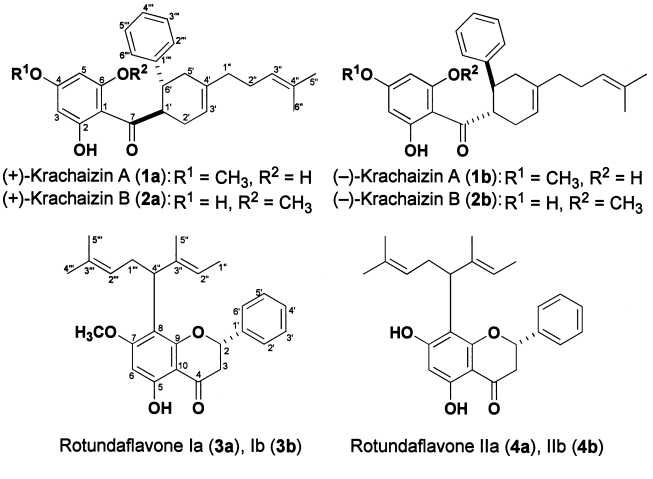

Chart 1

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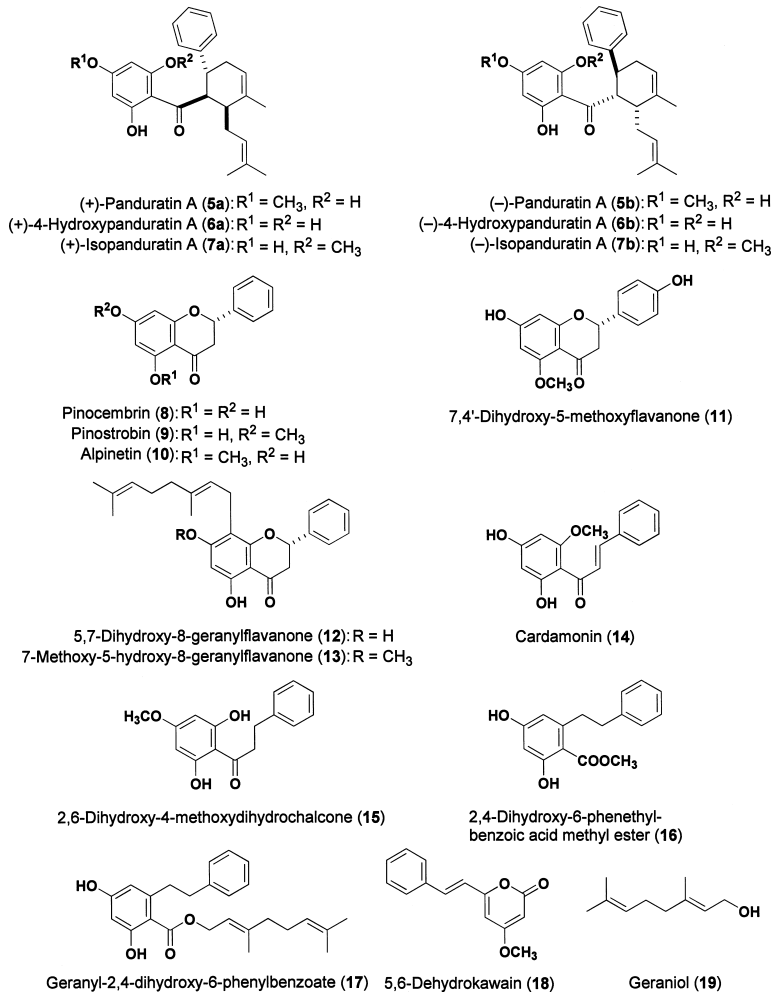

Chart 2

spectively. The electron ionization (EI)-MS of **1a** and **1b** showed the same molecular ion peak at *m/z* 406 (M^+), and the molecular formula $C_{26}H_{30}O_4$ was determined by high-resolution EI-MS measurement. The ^{1}H- ($CDCl_3$) and ^{13}C-NMR (Table 1) spectra of **1a** and **1b**, which were assigned by various NMR experiments,[20] showed the same signals assignable to two methyls [δ 1.59, 1.68 (3H each, both s, 6″, 5″-H_3)], four methylenes [δ 2.02, 2.08 (2H each, both m, 1″, 2″-H_2), 2.18, 2.25 (1H each, both m, 5′$_{ax}$, 5′$_{eq}$-H), 2.24, 2.60 (1H each, both m, 2′$_{ax}$, 2′$_{eq}$-H)], two methines [δ 3.32 (1H, m, 6′-H), 4.50 (1H, ddd-like, 1′-H)], two trisubstituted olefins [δ 5.10 (1H, dd-like, 3″-H), 5.51 (1H, br s, 3′-H)], seven aromatic protons [δ 5.85 (2H, br s, 3,5-H), 7.04—7.25 (5H, m, 2‴—6‴-H)], and a chelated hydroxyl proton [δ 12.20 (1H, br s, 2-OH)] together with a methoxyl group [δ 3.69 (3H, s, OCH_3)]. The common plannar structure to **1a** and **1b** was constructed on the basis of ^{1}H–^{1}H COSY and HMBC experiments. Thus, the ^{1}H–^{1}H COSY experiment on **1a** and **1b** indicated the presence of three partial structures shown as bold lines in Fig. 1. In the HMBC experiment, long-range correlations were observed between the following protons and carbons (3,5-H and 1-C; 1′-H and 7-C; 5′-H_2 and 3′, 4′-C; 6′-H and 1‴, 2‴,6‴-C; 1″-H_2 and 4′-C; 3″-H and 5″, 6″-C; 5″-H_3 and 3″, 4″, 6″-C; 6″-H_3 and 3″—5″-C; OCH_3 and 3,5, 4-C). The relative stereostructures of **1a** and **1b** were characterized by nuclear Overhauser and exchange spectroscopy (NOESY)

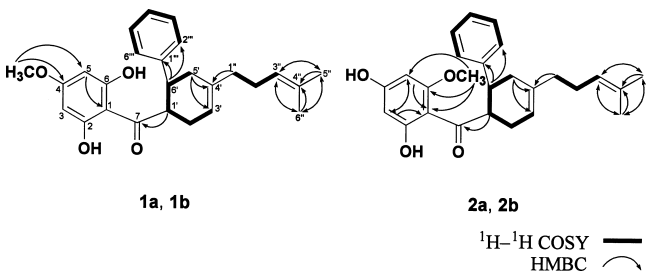

Fig. 1. ^{1}H–^{1}H COSY and HMBC Correlations of **1a**—**2b**

experiment, which showed NOE correlations between the following proton pairs (1′-H and 2′$_{eq}$, 5′$_{ax}$-H; 6′-H and 2′$_{ax}$, 5′$_{eq}$-H). By comparison of the NMR data of **1a** and **1b** with those of (±)-nicolaioidesin C,[21] the relative structures of **1a** and **1b** were confirmed. In the circular dichroic (CD) spectrum of **1a**, negative Cotton effects were observed at 226 nm ($\Delta\varepsilon$ +1.17), 283 nm (+1.24), and 347 nm (−0.05).[1] On the other hand, the CD spectrum of **1b** showed positive Cotton effects [221 nm ($\Delta\varepsilon$ −2.41), 285 nm (−1.35), and 338 nm (+0.18)]. On the basis of above-mentioned evidence, the absolute stereostructures of **1a** and **1b** were elucidated to be 1′*R*,6′*R* and 1′*S*,6′*S* orientations, respectively.

(+)-Krachaizin B (**2a**) and (−)-krachaizin B (**2b**) were isolated *ca.* 1 : 1 ratio with positive and negative optical rota-

Table 1. ^{13}C-NMR Data for **1a**—**2b**

Position	**1a**, **1b**	**2a**, **2b**
1	105.4	106.6
2	166.9	166.9
3	94.3	96.5
4	165.5	162.5
5	94.3	90.9
6	165.5	163.0
7	209.1	208.8
1′	49.9	50.4
2′	30.6	30.5
3′	119.2	119.4
4′	137.4	137.4
5′	38.3	38.1
6′	42.8	43.0
1″	37.3	37.3
2″	26.4	26.4
3″	124.1	124.2
4″	131.5	131.6
5″	25.7	25.7
6″	17.7	17.7
1‴	145.4	145.4
2‴, 6‴	127.2	127.3
3‴, 5‴	128.3	128.3
4‴	126.0	126.0
OCH_3	55.4	55.8

Measured in $CDCl_3$ at 125 MHz.

Table 2. ^{13}C-NMR Data for **3a**—**4b**

Position	**3a**	**3b**	**4a**	**4b**
2	79.31	79.28	79.09	78.92
3	43.60	43.59	43.56	43.47
4	195.86	195.85	195.86	195.80
5	161.54	161.56	162.01	162.02
6	91.11	91.11	96.12	96.24
7	166.59	166.59	165.23	165.21
8	111.89	111.86	108.61	108.59
9	161.37	161.37	161.23	161.16
10	102.85	102.87	102.63	102.67
1′	138.57	138.56	138.63	138.60
2′, 6′	126.16	126.16	126.12	126.15
3′, 5′	128.90	128.89	128.86	128.85
4′	128.90	128.89	128.80	128.78
1″	13.56	13.56	13.57	13.57
2″	117.45	117.44	120.54	120.54
3″	136.73	136.68	139.82	139.87
4″	41.97	41.92	40.99	41.05
5″	15.40	15.42	16.86	16.90
6″	28.93	28.98	29.35	29.30
7″	124.08	124.07	122.01	121.96
8″	131.22	131.23	132.79	132.80
9″	25.79	25.79	25.77	25.71
10″	17.81	17.78	17.73	17.69
OCH_3	55.69	55.70		

Measured in $CDCl_3$ at 125 MHz.

tions (**2a**: $[\alpha]_D^{26}$ +24°, **2b**: $[\alpha]_D^{25}$ −23° both in MeOH), respectively. The common molecular formula $C_{26}H_{30}O_4$ to **2a** and **2b** was determined from the molecular ion peak at *m/z* 406 (M^+) by EI-MS and high-resolution EI-MS measurements. The ^{1}H- ($CDCl_3$) and ^{13}C-NMR (Table 1) spectra[20] of **2a** and **2b** showed signals the same signals assignable to two methyls [δ 1.62, 1.70 (3H each, both s, 6″, 5″-H_3)], four methylenes [δ 2.02, 2.11 (2H each, both m, 1″, 2″-H_2), 2.13, 2.18 (1H each, both m, 5′$_{ax}$, 5′$_{eq}$-H), 2.23, 2.47 (1H each, both m, 2′$_{ax}$, 2′$_{eq}$-H)], two methines [δ 3.26 (1H, m, 6′-H), 4.25 (1H, m, 1′-H)], two trisubstituted olefins [δ 5.12 (1H, dd-like, 3″-H), 5.51 (1H, br s, 3′-H)], seven aromatic protons [δ 5.84, 5.87 (1H each, both br s, 3, 5-H), 7.04—7.22 (5H, m, 2‴—6‴-H)], and a chelated hydroxyl proton [δ 13.60 (1H, br s, 2-OH)] together with a methoxyl group [δ 3.88 (3H, s, OCH_3)], which were very similar to those of **1a** and **1b**. As shown in Fig. 1, the ^{1}H–^{1}H COSY experiment on **2a** and **2b** indicated the presence of three partial structures shown as bold lines. The long-range correlations in the HMBC experiment on **2a** and **2b** were observed between the following protons and carbons (3-H and 1, 5-C; 5-H and 1, 3-C; 1′-H and 7-C; 5′-H_2 and 3′, 4′-C; 6′-H and 1‴, 2‴,6‴-C; 1″-H_2 and 4′-C; 3″-H and 5″, 6″-C; 5″-H_3 and 3″, 4″, 6″-C; 6″-H_3 and 3″—5″-C; OCH_3 and 1, 5, 6-C). The NOE correlations of **2a** and **2b** in the NOESY experiment were observed between the following proton pairs (1′-H and 2′$_{eq}$, 5′$_{ax}$-H; 6′-H and 2′$_{ax}$, 5′$_{eq}$-H). The CD spectra of **2a** showed negative Cotton effects [289 nm (+2.74), 342 nm (−0.45)], whereas **2b** showed positive Cotton effects [288 nm (−2.49), 339 nm (+0.52)]. On the basis of above-mentioned evidence, the absolute stereostructures of **2a** and **2b** were determined to be 1′*R*,6′*R* and 1′*S*,6′*S* orientations, respectively.

Structures of Rotundaflavanones Ia (3a), Ib (3b), IIa (4a), and IIb (4b) Rotundaflavanones Ia (**3a**) and Ib (**3b**) were isolated as amorphous powders. The EI-MS of **3a** and **3b** showed the same molecular ion peak at *m/z* 406 (M^+) and the molecular formula $C_{26}H_{30}O_4$ was determined by high-resolution EI-MS measurement. The IR spectrum of **3a** and **3b** showed absorption bands at (**3a**: 1646, 1583, 1452, 1211, 1106 cm^{-1}, **3b**: 1644, 1592, 1456, 1202, 1103 cm^{-1}) ascribable to chelated carbonyl and olefin functions and benzene ring. In the UV spectrum of **3a** and **3b**, absorption maxima were observed at [**3a**: 294 (log ε 4.43), 340 (3.78), **3b**: 294 (4.53), 339 (3.88) nm], suggestive of the flavanone structure.[22—24] The ^{1}H- ($CDCl_3$) and ^{13}C-NMR (Table 2) spectra of **3a**, which were assigned by various NMR experiments,[20] showed signals assignable to a dihydropyrone moiety in flavanone structure by a characteristic ABX type coupling pattern [δ 2.79 (1H, dd, J=2.7, 17.1 Hz, 3$_{eq}$-H), 3.10 (1H, dd, J=13.4, 17.1 Hz, 3$_{ax}$-H), 5.42 (1H, dd, J=2.7, 13.4 Hz, 2-H)], a singlet aromatic proton [δ 6.06 (1H, s, 6-H)], monosubstituted benzene protons [δ 7.37—7.47 (5H, m, 2′—6′-H)], a chelated hydroxyl proton [δ 12.24 (1H, br s, 5-OH)] together with four methyls [δ 1.56, 1.58, 1.59 (3H each, all s, 5‴, 5″, 4‴-H_3), 1.60 (3H, d, J=6.8 Hz, 1″-H_3)], a methylene [δ 2.56, 2.67 (1H each, both m, 1‴-H_2)], a methine bearing an oxygen function [δ 3.80 (1H, dd-like, 4″-H)], two trisubstituted olefins [δ 5.00 (1H, dd, J=7.4, 7.6 Hz, 2‴-H), 5.36 (1H, q, J=6.8 Hz, 2″-H)], and a methoxyl group [δ 3.78 (3H, s, OCH_3)]. The proton and carbon signals in the ^{1}H- and ^{13}C-NMR (Table 2) spectra[20] ($CDCl_3$) of **3b** were found to be similar to those of **3a**. The connectivities of the quaternary carbons in **3a** and **3b** were clarified on the basis of the HMBC experiment. As shown in Fig. 2, the same long-range correlations were observed between the following proton and carbon pairs (2-H and 4, 1′, 2′,6′-C; 3-H_2 and 4, 10, 1′-C; 6-H and 8, 10-C; 2′,6′-H and 2-C; 1″-H_3 and 3″-C; 2″-H and 3″, 4″-C; 4″-H and 7, 2″, 3″-C; 5″-H_3 and 3″, 4″-C; 1‴-H_2 and 8-C; 2‴-H and 4‴, 5‴-C; 4‴-H_3 and 2‴, 3‴, 5‴-C; 5‴-H_3 and 2‴—4‴-C; OCH_3 and 7-C). On the basis of the above-men-

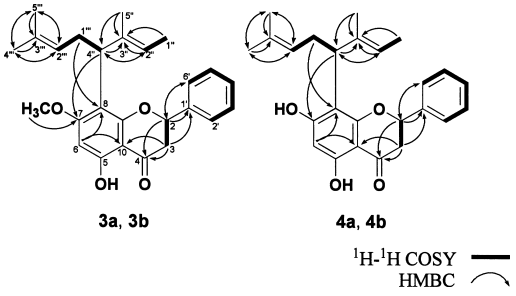

Fig. 2. ^{1}H–^{1}H COSY and HMBC Correlations of **3a**—**4b**

tioned evidence, the relative stereostructures of rotundaflavanones Ia (**3a**) and Ib (**3b**) were clarified except for the 4″-position.

The same molecular formula of rotundaflavanones IIa (**4a**) and IIb (**4b**), $C_{25}H_{28}O_4$, were observed by EI-MS and high-resolution EI-MS measurements, respectively. The ^{1}H- and ^{13}C-NMR (Table 3) spectra[20] ($CDCl_3$) of **4a** and **4b** showed signals assignable to four methyls [δ **4a**: 1.53, 1.60, 1.64 (3H each, all s, 5‴, 5″, 4‴-H_3), 1.73 (3H, d, J=6.4 Hz, 1″-H_3); **4b**: 1.49, 1.60, 1.64 (3H each, all s, 5‴, 5″, 4‴-H_3), 1.73 (3H, d, J=6.4 Hz, 1″-H_3)], a methylene [δ **4a**: 2.40, 2.53 (1H each, both m, 1‴-H_2); **4b**: 2.40, 2.53 (1H each, both m, 1‴-H_2)], a methine bearing an oxygen function [δ **4a**: 3.89 (1H, m, 4″-H); **4b**: 3.87 (1H, m, 4″-H)], two trisubstituted olefins [δ **4a**: 5.10 (1H, m, 2‴-H), 5.77 (1H, q, J=6.4 Hz, 2″-H); **4b**: 5.10 (1H, m, 2‴-H), 5.77 (1H, q, J=6.4 Hz, 2″-H)], a dihydropyrone moiety in flavanone structure by a characteristic ABX type coupling pattern [δ **4a**: 2.81 (1H, dd, J=3.0, 17.1 Hz, 3_{eq}-H), 3.09 (1H, dd, J=13.1, 17.1 Hz, 3_{ax}-H), 5.41 (1H, dd, J=3.0, 13.1 Hz, 2-H); **4b**: 2.84 (1H, dd, J=3.0, 17.1 Hz, 3_{eq}-H), 3.09 (1H, dd, J=13.1, 17.1 Hz, 3_{ax}-H), 5.41 (1H, dd, J=3.0, 13.1 Hz, 2-H)], a singlet aromatic proton [δ **4a**: 5.95 (1H, s, 6-H); **4b**: 5.95 (1H, s, 6-H)], monosubstituted benzene protons [δ **4a**: 7.37—7.47 (5H, m, 2′—6′-H); **4b**: 7.37—7.47 (5H, m, 2′—6′-H)], and a chelated hydroxyl proton [δ **4a**: 12.51 (1H, br s, 5-OH); **4b**: 12.51 (1H, br s, 5-OH)]. The proton and carbon signals in the ^{1}H- and ^{13}C-NMR (Table 2) spectra[20] ($CDCl_3$) of **4a** and **4b** were found to be similar to those of **3a** and **3b**, except for the lacking a methoxyl group. In the HMBC experiment of **4a** and **4b**, long-range correlations were observed as shown in Fig. 2. Thus, the relative stereostructures of rotundaflavanones IIa (**4a**) and IIb (**4b**) were clarified except for the 4″-position.

Effect on TNF-α-Induced Cell Death in L929 Cells TNF-α mediates a number of forms of organ injury through its induction of cellular apoptosis. In the case of liver, the biological effects of TNF-α have been implicated in hepatic injury induced by hepatic toxins, ischemia/reperfusion, vital hepatitis, and alcohol.[25—27] Therefore, TNF-α is considered an important target in research to discover anti-inflammatory and hepatoprotective agents. On the basis of above-mentioned concept, we investigated protective constituents from naturally occurring products on TNF-α-induced cell death in L929 cells, a TNF-α-sensitive cell line.[28] Previously, we have reported that an amide constituent piperine, which was isolated from the fruit of *Piper chaba*, was found to show TNF-α inhibitory activity.[14] The methanolic extract from the dried rhizomes of *B. rotunda* was found to inhibit on TNF-α-induced cytotoxicity in L929 cells [inhibition (%): 24±9 at 3 μg/ml, 83±11** at 10 μg/ml, IC_{50}=6.1 μg/ml].[29] To clarify the active constituents, we examined the effects of the constituents from *B. rotunda* on TNF-α-induced cytotoxicity in L929 cells. As shown in Table 3, (+)-krachaizin B (**2a**, IC_{50}=*ca.* 30 μM), (−)-krachaizin B (**2b**, 15 μM), (+)-4-hydroxypanduratin A (**6a**, *ca.* 10 μM), (−)-4-hydroxypanduratin A (**6b**, *ca.* 30 μM), (+)-isopanduratin A (**7a**, *ca.* 9 μM), (−)-isopanduratin A (**7b**, *ca.* 30 μM), alpinetin (**10**, 12 μM), cardamonin (**14**, 7 μM), and 2,6-dihydroxy-4-methoxydihydrochalcone (**15**, 23 μM), were found to show inhibitory activity, which were stronger than those of piperine (*ca.* 52 μM) and silybin (41 μM).

Effect on Aminopeptidase N Inhibitory Activity Aminopeptidase N (APN) is a Zn^+-dependent metalloprotease and plays an important role in tumor-cell invasion, extracellular matrix degradation by tumor cells and tumor metastasis.[30,31] Recently, APN was identified on the endothelial cell surface as a receptor for a tumor-homing peptide motif, NGR (asparagines-glycine-arginine, Asn-Gly-Arg), which is capable of homing selectively to the tumor vasculature.[32] This evidence indicated that APN plays a critical role in angiogenesis. Accordingly, APN is considered as an important therapeutic target for tumor angiogenesis and metastasis. Previously, we have reported the isolation and structure elucidation of several flavonoids with APN inhibitory activity from the whole plant of *Sinocrassula indica*.[33] In the course of our characterization studies on bioactive constituents from Thai natural medicines, inhibitory effect of the constituents from *B. rotunda* on APN activity was examined. As shown in Table 4, most of them except for geraniol (**19**) were found to inhibit APN activity at 10—30 μM, significantly. Especially, the APN inhibitory activity of (+)-krachaizin B (**2a**), (−)-krachaizin B (**2b**), (+)-panduratin A (**5a**), (−)-panduratin A (**5b**), (+)-4-hydroxypanduratin A (**6a**), (−)-isopanduratin A (**7b**), and geranyl-2,4-dihydroxy-6-phenylbenzoate (**17**) were found to show potent inhibitory activity (>50% inhibition at 30 μM), which were stronger than that of curcumin (31.9±1.6% at 30 μM).[34]

Experimental

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l=5 cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; FAB-MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer; ^{1}H-NMR spectra, JEOL JNM-LA500 (500 MHz) spectrometer; ^{13}C-NMR spectra, JEOL JNM-LA500 (125 MHz) spectrometer with tetramethylsilane as an internal standard; HPLC detector, Shimadzu RID-6A refractive index and SPD-10A*vp* UV–VIS detectors; and HPLC column, YMC-Pack ODS-A (YMC Co., Ltd. 250×4.6 mm i.d. and 250×20 mm i.d.) and Ceramospher Chiral RU-1 and RU-2 (Shiseido Co., Ltd. 250×4.6 mm i.d. and 250×10 mm i.d.) columns were used for analytical and preparative purposes, respectively.

The following experimental conditions were used for chromatography: ordinary-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150—350 mesh); reverse-phase silica gel column chromatography, Diaion HP-20 (Nippon Rensui) and Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh); TLC, precoated TLC plates with Silica gel $60F_{254}$ (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reverse phase); reverse-phase HPTLC, precoated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm); and detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

Plant Material The rhizomes of *B. rotunda* were cultivated in Nakhonsithammarat province, Thailand in April 2002, and were identified by Dr. Y. Pongpiriyadacha (Lecturer of Faculty of Science and Technology, Rajaman-

Table 3. Inhibitory Effects of Constituents from *B. rotunda* on TNF-α-Induced Cell Death in L929 Cells

	Inhibition (%)				
	0 μM	3 μM	10 μM	30 μM	100 μM
(+)-Krachaizin A (**1a**)	0±2	4±5	1±2	5±5	22±4*
(−)-Krachaizin A (**1b**)	0±3	3±1	1±1	−1±3	27±3**
(+)-Krachaizin B (**2a**)	0±4	13±7	15±5	54±0**	
(−)-Krachaizin B (**2b**)	0±5	21±6	38±2*	65±9**	
(+)-Panduratin A (**5a**)	0±3	6±9	−11±5		
(−)-Panduratin A (**5b**)	0±15	11±7	—	35±3*	
(+)-4-Hydroxypanduratin A (**6a**)	0±6	20±2*	49±9**	108±6**	
(−)-4-Hydroxypanduratin A (**6b**)	0±6	7±5	32±7**	48±1**	
(+)-Isopanduratin A (**7a**)	0±4	30±2**	53±7**		
(−)-Isopanduratin A (**7b**)	0±7	32±4**	46±4**		
Pinocembrin (**8**)	0±5	11±5	7±1	1±5	3±11
Pinostrobin (**9**)	0±5	−3±5	−5±5	13±13	29±6*
Alpinetin (**10**)	0±7	19±9	46±10**	76±4**	
7,4′-Dihydroxy-5-methoxyflavanone (**11**)	0±8	−10±7	3±4	4±5	24±5*
5,7-Dihydroxy-8-geranylflavanone (**12**)	0±5	−5±2	16±3	29±2**	27±14
7-Methoxy-5-hydroxy-8-geranylflavanone (**13**)	0±10	2±3	−4±6	−5±3	62±8**
Cardamonin (**14**)	0±4	16±8	73±8**		
2,6-Dihydroxy-4-methoxydihydrochalcone (**15**)	0±7	−10±6	14±5	66±14**	
2,4-Dihydroxy-6-phenethylbenzoic acid methyl ester (**16**)	0±7	2±5	−12±6	1±5	19±3
Geranyl-2,4-dihydroxy-6-phenylbenzoate (**17**)	0±6	−4±5	18±4	11±4	
5,6-Dehydrokawain (**18**)	0±10	4±5	−4±6	6±9	17±6
Geraniol (**19**)	0±4	5±5	−1±2	23±4	60±6**
Piperine	0±1	8±1	5±1	1±2	68±10**
Silybin[a)]	0±3	1±3	39±1**	45±3**	59±4**

Each value represents the mean±S.E.M. (n=4). Significantly different from the control, $*p<0.05$, $**p<0.01$. *a*) Commercial silybin was purchased from Funakoshi Co., Ltd. (Tokyo, Japan).

Table 4. Inhibitory Effects of Constituents from *B. rotunda* on Aminopeptidase N Activity

	Inhibition (%)		
	0 μM	10 μM	30 μM
(+)-Krachaizin A (**1a**)	0.0±3.6	6.8±1.2	27.9±0.6**
(−)-Krachaizin A (**1b**)	0.0±3.6	6.9±0.6	21.7±4.1**
(+)-Krachaizin B (**2a**)	0.0±1.1	44.8±1.7**	76.2±8.7**
(−)-Krachaizin B (**2b**)	0.0±3.6	27.8±3.3**	51.0±0.4**
(+)-Panduratin A (**5a**)	0.0±3.6	37.5±1.6**	61.4±1.4**
(−)-Panduratin A (**5b**)	0.0±1.3	62.1±1.2**	95.2±0.4**
(+)-4-Hydroxypanduratin A (**6a**)	0.0±1.3	32.5±1.8**	54.8±0.6**
(−)-4-Hydroxypanduratin A (**6b**)	0.0±1.3	25.1±1.3**	39.6±1.1**
(+)-Isopanduratin A (**7a**)	0.0±1.3	19.0±1.0**	29.3±1.7**
(−)-Isopanduratin A (**7b**)	0.0±1.1	44.5±1.9**	95.3±1.4**
Pinocembrin (**8**)	0.0±1.2	9.0±1.4*	13.3±2.4**
Pinostrobin (**9**)	0.0±1.2	14.5±3.1**	10.5±0.9**
Alpinetin (**10**)	0.0±1.2	13.4±2.1**	36.4±2.0**
7,4′-Dihydroxy-5-methoxyflavanone (**11**)	0.0±1.4	9.6±2.0	14.4±1.8**
5,7-Dihydroxy-8-geranylflavanone (**12**)	0.0±1.1	9.7±2.1	28.9±1.1**
7-Methoxy-5-hydroxy-8-geranylflavanone (**13**)	0.0±1.1	18.7±2.1**	17.0±2.7*
Cardamonin (**14**)	0.0±1.2	12.9±0.6**	37.2±1.7**
2,6-Dihydroxy-4-methoxydihydrochalcone (**15**)	0.0±0.7	7.6±0.6	14.8±1.0**
2,4-Dihydroxy-6-phenethylbenzoic acid methyl ester (**16**)	0.0±1.4	19.9±1.4**	22.8±1.9**
Geranyl-2,4-dihydroxy-6-phenylbenzoate (**17**)	0.0±1.4	56.4±1.8**	89.4±1.5**
5,6-Dehydrokawain (**18**)	0.0±0.7	5.2±1.5	17.7±2.1**
Geraniol (**19**)	0.0±1.4	7.2±1.6	7.6±1.5
Curcumin[33)]	0.0±1.7	13.3±0.9**	31.9±1.6**

Each value represents the mean±S.E.M. (n=4). Significantly different from the control, $*p<0.05$, $**p<0.01$.

gala University of Technology Srivijaya). A voucher specimen (No. T-08) is on file in our laboratory.[1)]

Extraction and Isolation Fractions 2-5 (58.4 mg), 2-7 (156.2 mg), 5-2-5 (149.9 mg), and 6-7-3 (54.7 mg) obtained from the methanolic extract of the dried rhizomes of *B. rotunda* (10.2% from the dried rhizomes) as reported previously.[1)] Fraction 2-5 (58.4 mg) was purified by HPLC [RI detector, YMC-Pack ODS-A, MeOH–H_2O (85 : 15, v/v) and UV detector (254 nm), Ceramospher Chiral RU-2, MeOH–H_2O (90 : 10, v/v)] to furnish rotundaflavanones IIa (**4a**, 3.8 mg, 0.00048%) and IIb (**4b**, 2.9 mg, 0.00037%). Fraction 2-7 (156.2 mg) was purified by HPLC [RI detector, YMC-Pack ODS-A, MeOH–H_2O (85 : 15, v/v) and UV detector (254 nm), Ceramospher Chiral RU-1, MeOH] to furnish rotundaflavanones Ia (**3a**, 8.2 mg, 0.0010%) and Ib

(**3b**, 19.3 mg, 0.0025%). Fraction 5-2-5 (149.1 mg) was purified by HPLC [UV detector (254 nm), Ceramospher Chiral RU-2, CH_3CN–H_2O (60 : 40, v/v)] to furnish (+)-krachaizin A (**1a**, 11.5 mg, 0.0015%) and (−)-krachaizin A (**1b**, 11.2 mg, 0.0014%). Fraction 6-7-3 (54.7 mg) was purified by HPLC [UV detector (254 nm), Ceramospher Chiral RU-1, MeOH] to furnish (+)-krachaizin B (**2a**, 17.0 mg, 0.0022%) and (−)-krachaizin B (**2b**, 13.8 mg, 0.0018%).

(+)-Krachaizin A (**1a**): A white powder, $[\alpha]_D^{22}$ +82° (c=0.11, MeOH). High-resolution EI-MS: Calcd for $C_{26}H_{30}O_4$ $(M)^+$: 406.2144. Found: 406.2141. CD [MeOH, nm ($\Delta\varepsilon$)]: 226 (+1.17), 283 (+1.24), 347 (−0.05). UV [MeOH, nm (log ε)]: 294 (3.65). IR (KBr): 3520, 1622, 1209 cm^{-1}. EI-MS m/z: 406 (M^+, 3), 388 (1), 271 (4), 167 (100).

(−)-Krachaizin A (**1b**): A white powder, $[\alpha]_D^{23}$ −78° (c=0.12, MeOH). High-resolution EI-MS: Calcd for $C_{26}H_{30}O_4$ $(M)^+$: 406.2144. Found: 406.2136. CD [MeOH, nm ($\Delta\varepsilon$)]: 221 (−2.41), 285 (−1.35), 338 (+0.18). UV [MeOH, nm (log ε)]: 295 (3.65). IR (KBr): 3500, 1641, 1207 cm^{-1}. EI-MS m/z: 406 (M^+, 3), 388 (1), 271 (3), 167 (100).

Compounds **1a** and **1b**: ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.59, 1.68 (3H each, both s, 6″, 5″-H_3), 2.02, 2.08 (2H each, both m, 1″, 2″-H_2), 2.18, 2.25 (1H each, both m, 5′$_{ax}$, 5′$_{eq}$-H), 2.24, 2.60 (1H each, both m, 2′$_{ax}$, 2′$_{eq}$-H), 3.32 (1H, m, 6′-H), 3.69 (3H, s, OCH_3), 4.50 (1H, ddd-like, 1′-H), 5.10 (1H, dd-like, 3″-H), 5.51 (1H, br s, 3′-H), 5.85 (2H, br s, 3, 5-H), 7.04—7.25 (5H, m, 2‴—6‴-H), 12.20 (1H, br s, 2-OH). ^{13}C-NMR data (125 MHz, $CDCl_3$) δ_C: given in Table 1.

(+)-Krachaizin B (**2a**): A white powder, $[\alpha]_D^{26}$ +24° (c=0.16, MeOH). High-resolution EI-MS: Calcd for $C_{26}H_{30}O_4$ $(M)^+$: 406.2144. Found: 406.2145. CD [MeOH, nm ($\Delta\varepsilon$)]: 214 (+5.46), 238 (−1.18), 289 (+2.74), 342 (−0.45). UV [MeOH, nm (log ε)]: 294 (4.11). IR (KBr): 3520, 1632, 1200 cm^{-1}. EI-MS m/z: 406 (M^+, 1), 167 (100).

(−)-Krachaizin B (**2b**): A white powder, $[\alpha]_D^{25}$ −23° (c=0.15, MeOH). High-resolution EI-MS: Calcd for $C_{26}H_{30}O_4$ $(M)^+$: 406.2144. Found: 406.2146. CD [MeOH, nm ($\Delta\varepsilon$)]: 216 (−5.61), 238 (+0.93), 288 (−2.49), 339 (+0.52). UV [MeOH, nm (log ε)]: 295 (4.07). IR (KBr): 3510, 1634, 1167 cm^{-1}. EI-MS m/z: 406 (M^+, 1), 167 (100).

Compounds **2a** and **2b**: ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.62, 1.70 (3H each, both s, 6″, 5″-H_3), 2.02, 2.11 (2H each, both m, 1″, 2″-H_2), 2.13, 2.18 (1H each, both m, 5′$_{ax}$, 5′$_{eq}$-H), 2.23, 2.47 (1H each, both m, 2′$_{ax}$, 2′$_{eq}$-H), 3.26 (1H, m, 6′-H), 3.88 (3H, s, OCH_3), 4.25 (1H, m, 1′-H), 5.12 (1H, dd-like, 3″-H), 5.51 (1H, br s, 3′-H), 5.84, 5.87 (1H each, both br s, 3, 5-H), 7.04—7.22 (5H, m, 2‴—6‴-H), 13.60 (1H, br s, 2-OH). ^{13}C-NMR data (125 MHz, $CDCl_3$) δ_C: given in Table 1.

Rotundaflavanone Ia (**3a**): An amorphous powder, $[\alpha]_D^{25}$ −47° (c=0.82, MeOH). High-resolution EI-MS: Calcd for $C_{26}H_{30}O_4$ $(M)^+$: 406.2144. Found: 406.2147. CD [MeOH, nm ($\Delta\varepsilon$)]: 287 (+6.66), 336 (−1.54). UV [MeOH, nm (log ε)]: 294 (4.43), 340 (3.78). IR (KBr): 1646, 1583, 1452, 1211, 1106 cm^{-1}. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.56, 1.58, 1.59 (3H each, all s, 5‴, 5″, 4‴-H_3), 1.60 (3H, d, J=6.8 Hz, 1″-H_3), 2.56, 2.67 (1H each, both m, 1‴-H_2), 2.79 (1H, dd, J=2.7, 17.1 Hz, 3$_{eq}$-H), 3.10 (1H, dd, J=13.4, 17.1 Hz, 3$_{ax}$-H), 3.78 (3H, s, OCH_3), 3.80 (1H, dd-like, 4″-H), 5.00 (1H, dd, J=7.4, 7.6 Hz, 2‴-H), 5.36 (1H, q, J=6.8 Hz, 2″-H), 5.42 (1H, dd, J=2.7, 13.4 Hz, 2-H), 6.06 (1H, s, 6-H), 7.37—7.47 (5H, m, 2′—6′-H), 12.24 (1H, br s, 5-OH). ^{13}C-NMR data (125 MHz, $CDCl_3$) δ_C: given in Table 2. EI-MS m/z: 406 (M^+, 2), 337 (96), 233 (100).

Rotundaflavanone Ib (**3b**): An amorphous powder, $[\alpha]_D^{25}$ −1° (c=0.42, MeOH). High-resolution EI-MS: Calcd for $C_{26}H_{30}O_4$ $(M)^+$: 406.2144. Found: 406.2148. CD [MeOH, nm ($\Delta\varepsilon$)]: 284 (+0.52), 328 (−0.21). UV [MeOH, nm (log ε)]: 294 (4.53), 339 (3.88). IR (KBr): 1644, 1592, 1456, 1202, 1103 cm^{-1}. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.55, 1.57, 1.60 (3H each, all s, 5‴, 5″, 4‴-H_3), 1.60 (3H, d, J=6.5 Hz, 1″-H_3), 2.56, 2.67 (1H each, both m, 1‴-H_2), 2.80 (1H, dd, J=3.1, 17.1 Hz, 3$_{eq}$-H), 3.10 (1H, dd, J=13.5, 17.1 Hz, 3$_{ax}$-H), 3.78 (3H, s, OCH_3), 3.80 (1H, m, 4″-H), 5.00 (1H, dd, J=6.7, 7.1 Hz, 2‴-H), 5.36 (1H, q, J=6.5 Hz, 2″-H), 5.42 (1H, dd, J=3.1, 13.5 Hz, 2-H), 6.06 (1H, s, 6-H), 7.37—7.47 (5H, m, 2′—6′-H), 12.24 (1H, br s, 5-OH). ^{13}C-NMR data (125 MHz, $CDCl_3$) δ_C: given in Table 2. EI-MS m/z: 406 (M^+, 2), 337 (99), 233 (100).

Rotundaflavanone IIa (**4a**): An amorphous powder, $[\alpha]_D^{26}$ +1° (c=0.42, MeOH). High-resolution EI-MS: Calcd for $C_{25}H_{28}O_4$ $(M)^+$: 392.1987. Found: 392.1993. CD [MeOH, nm ($\Delta\varepsilon$)]: 238 (−1.69), 253 (+0.94), 288 (+4.88), 332 (−0.78). UV [MeOH, nm (log ε)]: 291 (3.91). IR (KBr): 1636, 1466, 1154 cm^{-1}. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.53, 1.60, 1.64 (3H each, all s, 5‴, 5″, 4‴-H_3), 1.73 (3H, d, J=6.4 Hz, 1″-H_3), 2.40, 2.53 (1H each, both m, 1‴-H_2), 2.81 (1H, dd, J=3.0, 17.1 Hz, 3$_{eq}$-H), 3.09 (1H, dd, J=13.1, 17.1 Hz, 3$_{ax}$-H), 3.89 (1H, m, 4″-H), 5.10 (1H, m, 2‴-H), 5.41 (1H, dd, J=3.0, 13.1 Hz, 2-H), 5.77 (1H, q, J=6.4 Hz, 2″-H), 5.95 (1H, s, 6-H), 7.37—7.47 (5H, m, 2′—6′-H), 12.51 (1H, br s, 5-OH). ^{13}C-NMR data (125 MHz, $CDCl_3$) δ_C: given in Table 2. EI-MS m/z: 392 (M^+, 2), 323 (95), 219 (100), 149 (7).

Rotundaflavanone IIb (**4b**): An amorphous powder, $[\alpha]_D^{27}$ +7° (c=0.31, MeOH). High-resolution EI-MS: Calcd for $C_{25}H_{28}O_4$ $(M)^+$: 392.1987. Found: 392.1981. CD [MeOH, nm ($\Delta\varepsilon$)]: 242 (+0.91), 261 (+0.16), 286 (−1.47), 337 (+0.54). UV [MeOH, nm (log ε)]: 294 (3.91). IR (KBr): 1657, 1495, 1159 cm^{-1}. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.49, 1.60, 1.64 (3H each, all s, 5‴, 5″, 4‴-H_3), 1.73 (3H, d, J=6.4 Hz, 1″-H_3), 2.40, 2.53 (1H each, both m, 1‴-H_2), 2.84 (1H, dd, J=3.0, 17.1 Hz, 3$_{eq}$-H), 3.09 (1H, dd, J=13.1, 17.1 Hz, 3$_{ax}$-H), 3.87 (1H, m, 4″-H), 5.10 (1H, m, 2‴-H), 5.41 (1H, dd, J=3.0, 13.1 Hz, 2-H), 5.77 (1H, q, J=6.4 Hz, 2″-H), 5.95 (1H, s, 6-H), 7.37—7.47 (5H, m, 2′—6′-H), 12.51 (1H, br s, 5-OH). ^{13}C-NMR data (125 MHz, $CDCl_3$) δ_C: given in Table 2. EI-MS m/z: 392 (M^+, 2), 323 (29), 219 (27), 149 (100).

Bioassay Method. Inhibitory Effect on TNF-α-Induced Cell Death in L929 Cells Inhibitory effect on TNF-α-induced cell death in L929 cells was assayed by the method described in our previous report with slight modification.[14] Briefly, a suspension of 3×10^4 cells [obtained from Dainippon Pharmaceutical (Osaka, Japan)] in 100 μl of minimum essential medium Eagle supplemented with 1% non-essential amino acid solution (Invitrogen), FCS (10%), penicillin G (100 units/ml), and streptomycin (100 μg/ml) was incubated in a 96-well microplate. After 20 h of incubation in the medium containing actinomycin D (0.5 μg/ml) and TNF-α (40 pg/ml) with or without a test sample, the viability of the cells was assessed by the MTT colorimetric assay.[14] Silybin was purchased from Funakoshi Co., Ltd. (Tokyo, Japan).

Inhibitory Effect on Aminopeptidase N Inhibitory Activity Aminopeptidase N activity was assayed by the method described in a previous report.[33] Briefly, 0.2 mM of the enzyme substrate, L-alanine-4-methylcoumaryl-7-amide (Ala-MCA, Peptide Institute) in 50 mM Tris–HCl buffer containing 200 mM NaCl (pH 8.0, 100 μl/well) and sample solution (20 μl) in 96-well black microplates. The reaction mixture was initiated by adding an enzyme solution 100 μl/well (rat, 1 μU, Calbiochem) and incubated at 37 °C for 1 h to convert Ala-MCA to a fluorescent product, 7-amino-4-methylcoumarin (AMC). The reaction mixture was mixed with 0.1 M EDTA (50 μl/well) to stop the reaction. Fluorescence was measured using a fluorescence microplate reader (FLUOstar OPTIMA, BMG Labtechnologies) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Each sample was dissolved in dimethyl sulfoxide (DMSO) and diluted with PBS (final DMSO concentration in the incubation mixture was 0.1%). Curcumin was isolated from Thai Zedoary (the rhizomes of *Curcuma zedoaria* originating in Thailand)[15] and used as a reference compound.

Statistics Values were expressed as means±S.E.M. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical analysis. Probability (p) values less than 0.05 were considered significant.

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