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Ixorapeptide I and ixorapeptide II, bioactive peptides isolated from *Ixora coccinea*

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ABSTRACT

Two novel derivatized peptides, designated as ixorapeptide I (**1**) and ixorapeptide II (**2**), in addition to 28 other known compounds, were isolated from the MeOH extract of *Ixora coccinea* using bioassay-guided fractionation. The structures of metabolites **1** and **2** were determined by interpretation of the spectroscopic data and Marfey's method. Compound **1** exhibited selective potency against Hep3B liver cancer cell line with an IC_{50} value of 3.36 μg/mL, and compound **2** did not show notable cytotoxicity toward cancer cell lines but could inhibit superoxide anion generation and elastase release with IC_{50} values of 0.21 and 0.27 μg/mL, respectively. Moreover, kaempferol and luteolin from this plant showed inhibition with IC_{50} values of 3.55 and 2.56 μg/mL, respectively on platelet aggregation induced by collagen.

Ixora, a class of shrubs and small trees belonging to the Rubiaceae, is distributed in tropical Asia and Africa.[1] *Ixora coccinea* Linn., with small beautiful red flowers scattering on the outward of the plant body, is easily cultured and is one of the most common roadside and garden trees in Taiwan. In previous studies, the EtOH and MeOH extracts of *I. coccinea* showed antibacterial, antifungal,[2] antioxidative[3] and anti-tumor activities.[4] In addition, the water extract exhibited antioxidative[5] and analgesia activities.[6] However, only eight compounds, including five triterpenoids (oleanolic acid,[7] ursolic acid,[7,8] lupeol,[7,9] cycloartenol palmitate,[10] and cycloartenol tetradecanoate[10]) and three fatty acids (octadecanoic acid, octadecadienoic acid, and linoleic acid),[7] have been isolated from this plant. In an effort to discover natural anti-platelet aggregation and anti-inflammatory agents, we determined that the MeOH extract of this roadside plant showed significant activities toward both target functions without considerable cytotoxicity at a concentration of 20 μg/mL.

Bioassay-guided fractionation of aerial parts of *I. coccinea* led to the isolation of 30 compounds, including eight triterpenoids (lupeol, 3-acetyl betulic acid, betunolic acid, α-amyrin, β-amyrin, ursolic acid, 3-acetyl ursolic acid, and oleanonic acid), four steroids (6β-hydroxystigmast-4-en-3-one, sitosteryl-3-*O*-β-D-glucoside, β-sitosterol and stigmasterol mixture), seven flavonoids (kaempferol, kaempferol-7-*O*-α-rhamnoside, kaempferitrin, luteolin, (−)-*epi*-catechin, (+)-catechin, and epicatechin-(4β→8,2β→*O*→7)-*ent*-epicatechin), three coumarins (scopoletin, coumarin, and *erythro*-1′,2′-albiflorin), two diterpenoids (16α-hydro-19-acetoxy-(−)-kauran-17-oic acid and 16α-hydro-19-ol-(-)-kauran-17-oic acid), two peptides (ixorapeptide I and II (**1**; 10.10 mg and **2**; 11.30 mg)), two quinones (1,4-dihydroxy-3-methyl-anthraquinone and α-tocopheryl quinone), one triglycerol (2,3-dihydroxypropyl-eicosanoic ester) and one fatty acid (oleic acid) (see Fig. S1). Among these compounds, two novel derivatized peptides (**1** and **2**) were identified (Fig. 1). The majority of the compounds were evaluated in inflammation, platelet aggregation and cytotoxic assays, and the results of these experiments are described herein.

The MeOH extract of the aerial parts of *I. coccinea* was partitioned with $CHCl_3$/water (1:1), followed by further partitioning of the $CHCl_3$ and H_2O layers with 90% MeOH/*n*-hexane (1:1) and

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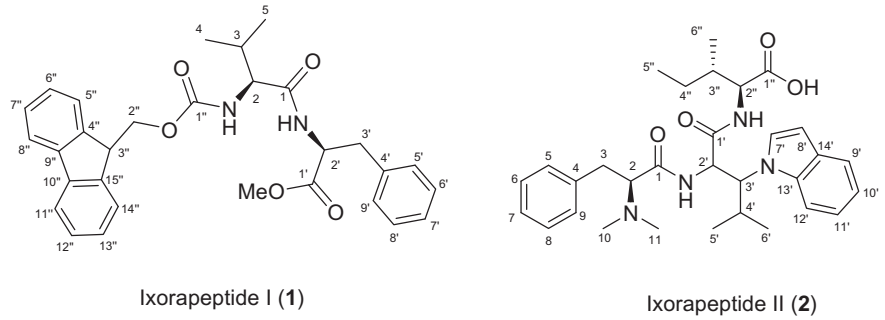

Figure 1. The structures of ixorapeptide I (**1**) and ixorapeptide II (**2**).

EtOAc/H_2O (1:1), respectively. The EtOAc and 90% MeOH fractions showed better anti-platelet aggregation and anti-inflammatory activity. Both active layers were investigated, and the novel peptides **1** and **2** were isolated from the later extract. Fractionation of the 90% MeOH extract was performed with liquid chromatography on silica gel using a gradient of CH_2Cl_2/MeOH to yield 10 subfractions. Si gel column chromatography on subfraction 4 (EtOAc/*n*-hexane 1:1) followed by purification led to the isolation of the two peptides.

HRESIMS of compound **1** exhibited a sodium-adduct $[M+Na]^+$ ion at *m/z* 523.2206 (calcd for $C_{30}H_{32}N_2O_5Na$), from which 16 degrees of unsaturation were deduced. The IR spectrum showed absorptions for amine (3282 cm^{-1}), the carbonyl of esters and amides (1728 and 1685 cm^{-1}), and aromatic (1651 and 1533 cm^{-1}) functional groups. In the 1D NMR spectra (Table 1), 30 carbon signals were observed, which were consistent with one methoxy, two methyl, two methylene, seventeen methine and eight quaternary carbons (including two carbonyl carbons at δ 170.7 and 171.7). 1D NMR and HMQC data indicated the presence of 13 aromatic protons (δ_H 7.09 (2H, d, J = 7.2 Hz, δ_C 129.1), 7.23 (2H, m, δ_C 127.1), 7.20 (1H, m, δ_C 128.6), 7.31 (2H, dt, J = 6.8, 2.4 Hz, δ_C 127.0), 7.39 (2H, t, J = 6.8 Hz, δ_C 127.7), 7.57 (2H, dd, J = 6.8, 2.4 Hz, δ_C 125.0), and 7.77 (2H, d, J = 6.8 Hz, δ_C 119.9)) and five quaternary aromatic carbons (δ_C 135.6, 141.2 (2C), 143.6 (2C)) that contributed to three aromatic rings. Additionally, two amide protons appeared in the ^1H NMR spectrum at δ 5.31 (d, J = 8.4 Hz) and 6.33 (d, J = 6.8 Hz). On the basis of COSY and HMBC data (Fig. 2), we speculated that compound **1** was composed of one valine, one methyl-phenylalanine and the fluorene moieties. To fulfill the sixteen degrees of unsaturation, nine degrees of unsaturation of fluorene and six degrees of two peptides indicated the existence of one additional degree. The remaining degree was assigned as δ_C 156.3 for a special carbamic moiety that linked between fluorene and valine functions with HMBC correlations of H-2 (δ 4.03)/C-1″ (δ 156.3), and H-2″ (δ 4.34, 4.42)/C-1″ (δ 156.3) (Fig. 2). The linkage of the valine and phenylalanine moieties was confirmed by the key HMBC correlation of H-2′ (δ 4.89)/C-1 (δ 170.7). To determine the absolute configuration, we treated compound **1** with 6 N HCl and Marfey reagent[11] and compared the HPLC retention time between the L-form amino acid standards (Fig. S14) and the L-valine and L-methyl-phenylalanine in compound **1**. The structure of compound **1** was determined to be Fmoc-L-Val-L-Phe-OMe. The structure was synthesized by Dr. Lorca and Kurosu in 2001 while experimenting with amide-forming reactions using in situ phosphonium salts without a tertiary amine.[12] However, clear spectral data and biological properties of synthesized **1** were never reported. Compound **1**, which we isolated is a naturally occurring new chemical and named as ixorapeptide I.

Table 1
^1H and ^{13}C NMR data for compound **1** (400 and 100 MHz in $CDCl_3$, δ in ppm, J in Hz)

Position	1	
	δ_H (J in Hz)	δ_C
1		170.7
2	4.03 dd (8.4, 5.6)	60.1
3	2.03 m	30.9
4	0.80 (3H) d (6.8)	17.9
5	0.86 (3H) d (6.8)	19.1
1′		171.7
2′	4.89 q (6.8)	52.7
3′a	3.06 dd (14.0, 6.8)	38.0
3′b	3.14 dd (14.0, 6.8)	
4′		135.6
5′, 9′	7.09 (2H) d (7.2)	129.1
6′, 8′	7.23 (2H) m	127.1
7′	7.20 m	128.6
1″		156.3
2″a	4.34 dd (10.4, 6.4)	67.1
2″b	4.42 dd (10.4, 6.4)	
3″	4.20 t (6.4)	47.1
4″, 15″		143.6
5″, 14″	7.57 (2H) dd (6.8, 2.4)	125.0
6″, 13″	7.31 (2H) dt (6.8, 2.4)	127.0
7″, 12″	7.39 (2H) t (6.8)	127.7
8″, 11″	7.77 (2H) d (6.8)	119.9
9″, 10″		141.2
OCH_3	3.71 (3H) s	52.4
NH	5.31 d (8.4)	
NH	6.33 d (6.8)	

An $[M+Na]^+$ ion at *m/z* 557.3663 (calcd for $C_{31}H_{42}N_4O_4Na$) was found in the HRESIMS of compound **2**. The IR spectrum showed absorptions for amine (3248 cm^{-1}), acid (3500–2500 cm^{-1}), carbonyl (1631 cm^{-1}) and aromatic (1514 cm^{-1}) functional groups. Thirty one carbon signals, consisting of six methyl, two methylene, 17 methine and six quaternary carbons, were observed in the 1D NMR data (Table 2). The three quaternary carbons were identified as carbonyl carbons due to their chemical shifts of δ_C 169.3, 171.1

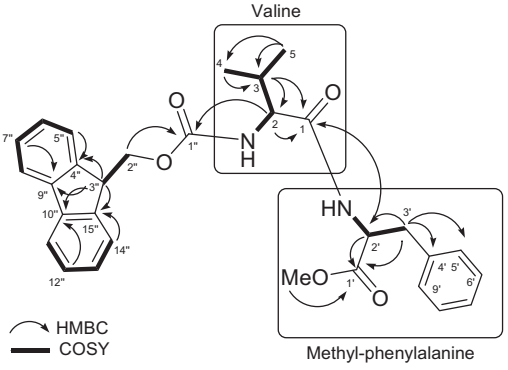

Figure 2. Key COSY and HMBC correlations of compound **1**.

Table 2
^{1}H and ^{13}C NMR data for compound **2** (600 and 150 MHz in C_5D_5N, δ in ppm, J in Hz)

Position	2	
	δ_H (J in Hz)	δ_C
1		171.1
2	3.70 t (6.6)	70.5
3a	3.14 dd (7.8, 6.6)	32.8
3b	3.34 dd (7.8, 6.6)	
4		140.6
5, 9	7.38 (2H) d (7.2)	129.5
6, 8	7.29 (2H) t (7.2)	128.6
7	7.16 t (7.2)	126.2
1′		172.1
2′	5.21 t (10.2)	56.0
3′	5.27 dd (10.2, 1.2)	81.1
4′	2.50 m	140.1
5′	1.24 (3H) d (7.2)	15.2
6′	1.20 (3H) d (7.2)	20.4
7′	6.79 d (8.2)	128.0
8′	6.58 m	127.5
9′	7.11 dd (8.4, 1.8)	130.8
10′	7.31 br t (8.4)	118.1
11′	7.24 m	130.1
12′	6.82 dd (8.4, 1.8)	121.1
13′		156.7
14′		131.4
1″		169.3
2″	4.55 t (9.0)	58.3
3″	2.01 m	36.4
4″a	1.12 m	24.7
4″b	1.48 m	
5″	0.65 (3H) t (7.2)	10.9
6″	0.82 (3H) d (7.2)	15.7
N–CH_3	2.41 (6H) s	41.6
C2′–NH	8.97 d (10.2)	
C2″–NH	9.07 br s	

and 172.1. The ^{1}H NMR spectrum of compound **2** showed two amide protons at δ 8.97 and 9.07. The investigation of COSY and HMBC spectra identified two amino acids, including isoleucine and phenylalanine (Fig. 3). The presence of two methyl groups (δ_H 2.41, 6H) possessing a HMBC correlation with Cα (δ 70.5) of phenylalanine suggested that this moiety is *N*,*N*-dimethyl phenylalanine (Fig. 3). As shown in Fig. 3, the fragment NH–CHCO–CH–CH–$(CH_3)_2$ was found, and the low field chemical shifts at δ_H 5.27 and δ_C 81.1 of the C-3′ methine indicated the linkage of a heteroatom. From the deduction of the above mentioned moieties of isoleucine, *N*,*N*-dimethyl phenylalanine and NH–CHCO–CH–CH–$(CH_3)_2$ from the total molecular formula, the residual elements were C_8H_6N. The 2D NMR spectroscopic analysis suggested the presence of an indole moiety. The HMBC correlations of H-2′/C-1, C-1′, H-3′/C-1′, and H-2″/C-1′ suggested linkages between the identified moieties (Fig. 3) and the plane structure was confirmed. To determine the absolute configuration, Marfey's method[11] was used and resulted in the assignment of L-isoleucine and L-*N*,*N*-dimethyl phenylalanine in compound **2** (Fig. S14). Although the stereochemistry of Cα of the unusual 3-indol leucine moiety was not confirmed directly by the authentic sample, it is predicted to have an L-leucine partial structure, similar to the other amino acid moieties found in this study. Thus, compound **2** was named ixorapeptide II.

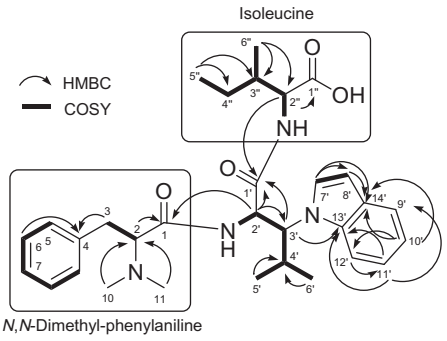

Figure 3. Key COSY and HMBC correlations of compound **2**.

The majority of the purified compounds were screened in a cytotoxicity assay using doxorubicin as the positive control. Only compound **1** exhibited selective potency against the Hep3B liver cancer cell line with an IC_{50} value of 3.36 μg/mL. The remaining compounds were inactive for each of the cancer cell lines (IC_{50} >4 μg/mL; Table S1, Supplementary data). The effects of the bioactivity-guided fractionated extracts on platelet aggregation were determined. Each isolated compound was subjected to a collagen-dependent platelet aggregation assay (Table S3). The results indicated that flavonoids are the major active ingredient of *I. coccinea*. Among the seven flavonoids, kaempferol and luteolin showed more significant inhibition than aspirin (IC_{50} 13.58 μg/mL) with IC_{50} values of 3.55 and 2.56 μg/mL, respectively. In a simple structure–activity relationship (SAR) study, we concluded that flavonone or flavononol was more favorable than flavane, and the flavonoids that have rhamnosyl functionality remarkably reduced the inhibitory activity of collagen-induced aggregation.

Interestingly, using an anti-inflammatory assay (Table S2), compound **2**, which did not have notable cytotoxicity in the cancer cell lines, inhibited superoxide anion generation and elastase release with IC_{50} values of 0.21 and 0.27 μg/mL, respectively. Compound **2** was a 73-fold more potent inhibitor of elastase release than the commercial reference drug, phenylmethylsulfonyl fluoride (PMSF). In addition, compound **2** showed similar potency to ursolic acid,[13] with IC_{50} values of 0.29 and 0.31 μg/mL for the inhibition of superoxide anion generation and elastase release, respectively.

Overall, our data demonstrate that the extracts of *I. coccinea* have significant anti-platelet aggregation effects. In addition, we report, for the first time, that ixorapeptide I (**1**)[14] and ixorapeptide II (**2**)[15] are novel bioactive natural products. Of note, peptide **2** showed significant anti-inflammatory effects on neutrophils.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.10.058.

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14. Ixorapeptide I (**1**): colorless needles; mp 182–184 °C; $[\alpha]_D^{23}$ −50.1 (c 0.6, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 210 (4.52), 264 (4.17), 300 (3.67); IR (neat) ν_{max} 3282, 1728, 1685, 1651, 1533 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Table 1; HRESIMS *m/z* 523.2206 (calcd for $C_{30}H_{32}N_2O_5Na$ 523.2207).
15. Ixorapeptide II (**2**): colorless needles; mp 291–293 °C; $[\alpha]_D^{23}$ −57.7 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 232 (4.23); IR (neat) ν_{max} 3248, 3500–2500, 1631, 1514 cm^{-1}; ^1H and ^{13}C NMR (C_5D_5N), see Table 2; HRESIMS *m/z* 557.3663 (calcd for $C_{31}H_{42}N_4O_4Na$ 557.3664).