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CYP3A4 Inhibitory Activity of New Bisalkaloids, Dipiperamides D and E, and Cognates from White Pepper

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Abstract—Two new bisalkaloids, dipiperamides D and E, were isolated as inhibitors of a drug metabolizing enzyme cytochrome P450 (CYP) 3A4 from the white pepper, *Piper nigrum*. Their structures were elucidated by spectroscopic methods. Dipiperamides D and E showed potent CYP3A4 inhibition with IC_{50} values of 0.79 and 0.12 μM, respectively, and other metabolites from the pepper were moderately active or inactive.

Introduction

Cytochrome P450 (CYP) enzymes are responsible for drug metabolism and constitute three families, CYP1, CYP2, and CYP3.[1] In human liver microsomes CYP3A4 is the most abundant enzyme, and recent investigations revealed that more than 50% of clinically used drugs are oxidized by CYP3A4.[2,3] Since CYP3A4 inhibitors may change the pharmacokinetics and bioavailability of concomitant drugs, by affecting their metabolism in humans, the search for CYP inhibitors in the diet is important for clinical therapeutics. In the course of our study of CYP inhibitors, we reported the isolation and structure elucidation of furanocoumarins from grapefruit juice[4,5] and bisalkaloids, dipiperamides A–C (**1**–**3**), from the white pepper *Piper nigrum*.[6] This paper reports the isolation, structure elucidation, and CYP inhibitory activity of two new bisalkaloids, dipiperamides D (**4**) and E (**5**), as well as 16 constituents in the white pepper, brachyamide A (**6**),[7] piperamide-C9:1(8*E*) (**7**),[8] piperamide-C9:3(2*E*,4*E*,8*E*) (**8**),[8] piperylin (**9**),[8] piperolein-B (**10**),[8] piperolein-A (**11**),[9] piperine (**12**),[8] *N*-*trans*-cinnamoylpiperidine (**13**),[10] guineensine (**14**),[8] pipercide (**15**),[7] retrofractamide A (**16**),[7] (2*E*,4*E*)-*N*-isobutyldecadienamide (**17**),[11] 2,4-decadienoylpiperidine (**18**),[12] *N*-*trans*-feruloylmethoxytyramine (**19**),[13] and *N*-*trans*-feruloyltyramine (**20**)[13] as well as (−)-hinokinin (**21**)[14] (Chart 1).

Results and Discussion

Isolation of dipiperamides D (4) and E (5)

The white pepper (1.0 kg) was refluxed for 1 h in acetone followed EtOAc extraction under reflux. The bioassay-monitored isolation of the combined extracts by silica gel and ODS chromatography followed by reverse-phase HPLC afforded dipiperamide D (**4**, 4.5 mg). The fractionation of independently prepared acetone extract from the white pepper (5.0 kg) afforded dipiperamide E (**5**, 3.5 mg).

Structure elucidation of dipiperamide D (4)

Dipiperamide D (**4**) was analyzed for $C_{36}H_{40}N_2O_6$ on the basis of its HREIMS. The ^1H and ^{13}C NMR spectral features (Table 1) suggested **4** was a congener of bisalkaloids, dipiperamides A–C (**1**–**3**),[6] by comparison with those of **1**–**3**. The molecular formula of **4** contains C_2H_2 excessive elements than those of **1** and **2**. The presence of an additional *trans* olefine group was indicated by the ^1H NMR data; δ 6.18 (dd, $J=15.6$, 8.8 Hz, H-4)/δ 6.39 (d, $J=15.6$ Hz, H-5), δ 6.30 (d, $J=15.6$ Hz, H-2″)/δ 6.82 (dd, $J=15.6$, 6.8 Hz, H-3″), and δ 6.15 (dd, $J=15.6$, 8.8 Hz, H-6″)/δ 6.29 (d, $J=15.6$ Hz, H-7″). The

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COSY spectrum of **4** suggested the sequential correlations from the former set due to H-2″/H-3″ to H-4″ (δ 3.36)/H-2 (δ 3.45)/H-3(δ 3.89)/H-5″ (δ 3.35). H-3 and H-5″ were found to correlate with the second and the third set of olefin signals due to H-4/H-5 and H-6″/H-7″, respectively (Fig. 1). HMBC cross peaks H-3″/C-5″ and H-6″/C-4″ implied the presence of a cyclobutane ring in **4** (Fig. 1). The positions of two methylenedioxyphenyl and two carbonyl groups were indicated by HMBC correlations; δ 3.36 (H-4″), 3.45 (H-2), 3.89 (H-3)/δ 169.1 (C-1); δ 6.18 (H-4), 6.39 (H-5)/δ 131.7 (C-6); δ 6.30 (H-2″), 6.82 (H-3″)/δ 165.2 (C-1″); δ 6.15 (H-6″), 6.29 (H-7″)/δ 131.8 (C-8″) (Fig. 1). Although NOESY experiment in $CDCl_3$ was performed to determine the relative stereochemistry of **4**, severely overlapping signals in δ 3.2–3.9 hampered the analysis of the NOE correlation. NOE difference sepctra could be measured in pyridine-d_5 because of the better separation of hydrogen signals in pyridine-d_5 better than in $CDCl_3$. NOE correlations, H-2 (δ 3.89, dd, $J=8.3$, 7.8 Hz)/H-4″ (δ 3.63, dt, $J=7.8$, 8.3 Hz,), H-3 (δ 4.32, q, $J=7.8$ Hz)/H-5″ (δ 3.47, q, $J=7.8$ Hz), H-2/H-4 (δ 6.57, dd, $J=16.1$, 7.8 Hz), H-4″/H-6″ (δ 6.62, dd, $J=15.6$, 7.8 Hz), and H-5″/H-3″ (δ 7.41, dd, $J=15.1$, 8.3 Hz), suggested a head-to-head structure for **4** as shown in Figure 2.[15]

Structure elucidation of dipiperamide E (5)

Dipiperamide E (**5**) has the same molecular formula $C_{34}H_{38}N_2O_6$ as **1** and **2**, and the ^1H and ^{13}C NMR data

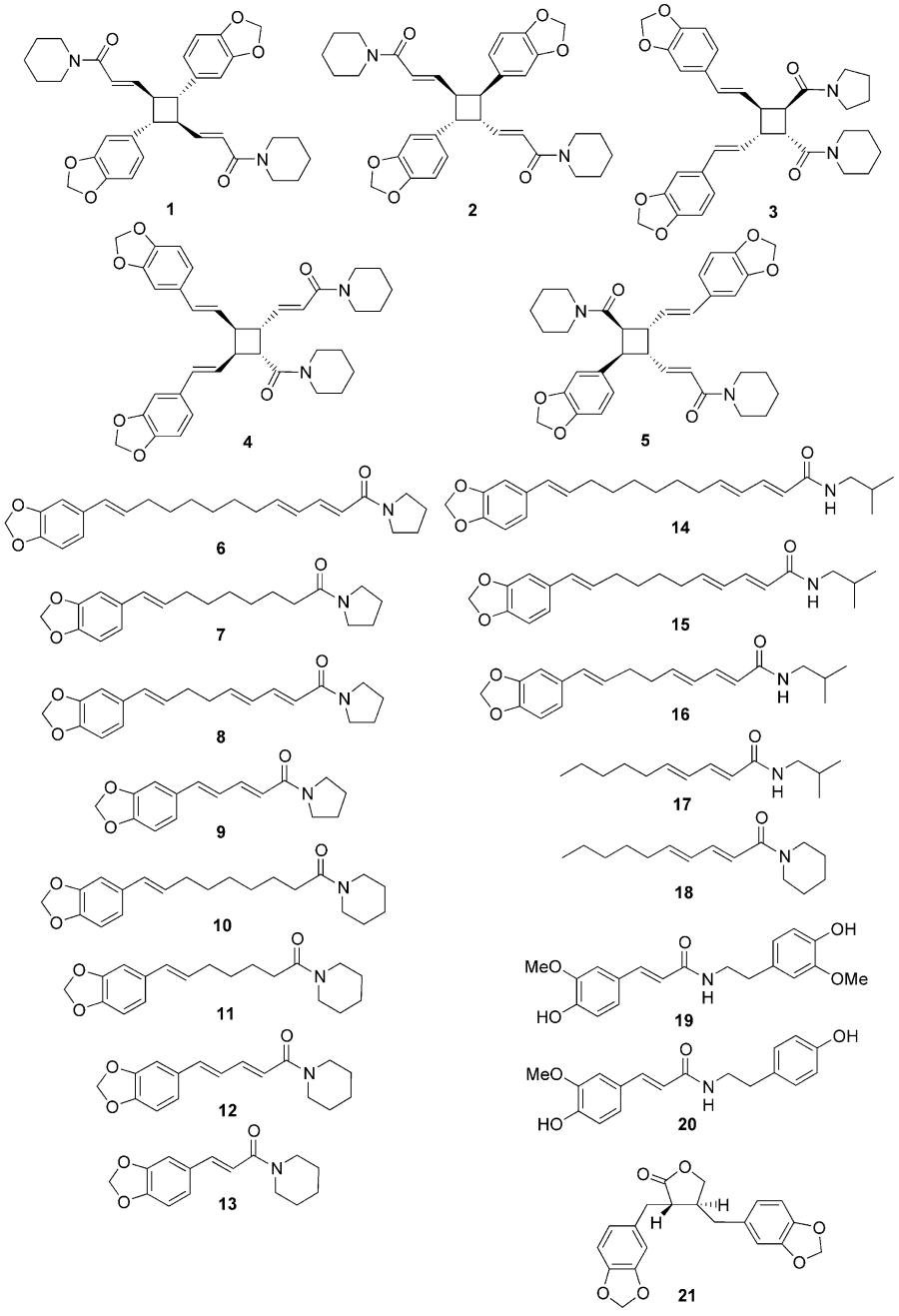

Chart 1.

of **5** (Table 2) were indicated to be an asymmetric bisalkaloid similar to **4**. The COSY spectrum of **5** revealed the presence of a cyclobutane ring (–C-4–C-5–C-2″–C-3″–) and *trans* olefin groups, C-3–C-2 and C-4″–C-5″, which were bonded to C-4 and C-3″, respectively (Fig. 3). HMBC cross peaks δ 3.78 (H-5)/δ 108.5 (C-7), δ 3.61 (H-4)/δ 133.3 (C-6), δ 6.29 (H-4″)/δ 131.7 (C-6″), and δ 6.43 (H-5″)/δ 105.7 (C-7″) suggested two methylenedioxyphenyl groups were bonded to C-5 and C-5″ (Fig. 3). The presence of carbonyl groups at C-2 and C-2″ was implied by HMBC cross peaks δ 6.16 (H-2) and 6.90 (H-3)/δ 165.2 (C-1) and δ 3.97 (H-3″) and 3.78 (H-5)/δ 169.5 (C-1″). The relative stereochemistry of **5** was determined by NOE difference spectra; the irradiation at δ 3.78 (H-5) caused enhancement of the signals at δ 6.90 (H-3) and 3.50 (H-2″), and the irradiation at δ 3.97 (H-3″) increased the signal at δ 3.61 (H-4) (Fig. 4). Thus, the stereostructure of **5** was established as shown in Chart 1.[15]

Table 1. NMR spectral data[a] for dipiperamide D (**4**) in $CDCl_3$

	δ_H	*J* (Hz)	δ_C		δ_H	*J* (Hz)	δ_C
1			169.1 s	2′	3.44	m	42.7 t
2	3.45	m	44.6 d		3.66	m	
3	3.89	q, 8.8	41.2 d	3′	1.55 (2H)	m	25.6 t
4	6.18	dd, 15.6, 8.8	127.6 d	4′	1.63 (2H)	m	24.5 t
5	6.39	d, 15.6	131.1 d	5′	1.55 (2H)	m	26.3 t
6			131.7 s	6′	3.24	m	46.2 t
7	6.87	d, 1.5	105.5 d		3.34	m	
8			147.9 s	2‴	3.46	m	43.1t
9			146.9 s		3.58	m	
10	6.72	d, 7.8	108.2 d	3‴	1.55 (2H)	m	25.5 t
11	6.74	dd, 7.8, 1.5	120.7 d	4‴	1.63 (2H)	m	24.6 t
1″			165.2 s	5‴	1.55 (2H)	m	26.6 t
2″	6.30	d, 15.6	122.1 d	6‴	3.46	m	47.1 t
3″	6.82	dd, 15.6, 7.8	142.2 d		3.58	m	
4″	3.36	m	44.2 d				
5″	3.35	m	45.4 d				
6″	6.15	dd, 15.6, 8.8	127.5 d				
7″	6.29	d, 15.6	130.9 d				
8″			131.8 s				
9″	6.87	d, 1.5	105.5 d				
10″			148.0 s				
11″			147.0 s				
12″	6.72	d, 7.8	108.2 d				
13″	6.74	dd, 7.8, 1.5	120.8 d				
OCH_2O×2	5.92 (4H)	s	100.9 s				
			101.0 s				

[a]Data for piperidine rings (2′-6′ and 2″-6″) may be interchangeable.

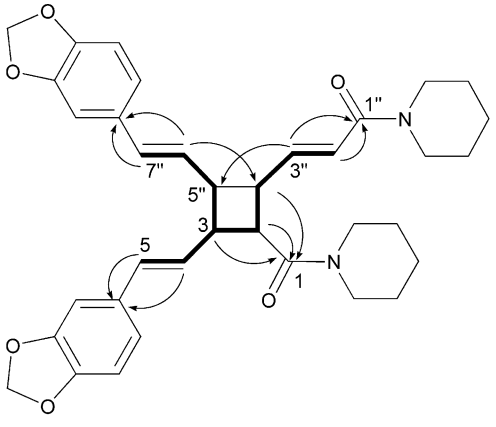

Figure 1. COSY (bold lines) and selected HMBC (arrows) correlations observed for dipiperamide D (**4**).

CYP3A4 inhibition of pepper constituents

CYP3A4 activity monitored by nifedipine oxidation with expressed human CYP3A4[4] varied depending on the size and function of pepper constituents. The major constituent piperine (**12**) consists of three components, that is, a methylenedioxyphenyl ring, side chain, and acylpiperidine ring. Among the pepper constituents we isolated, bisalkaloids (**1**–**5**) exhibited the most potent inhibition of CYP3A4 with IC_{50} values of less than 1 μM (Table 3). Although compounds bearing a pyrrolidine ring (**6**–**9**) showed moderate activity with IC_{50} values of 2–5 μM, activities of those containing a piperidine ring (**10**–**13**) varied with the length of the side chain: **12** and **13** having shorter side chains were less active than **10** and **11**. Compounds lacking a methylenedioxyphenyl ring (**17**–**20**) and those possessing an *iso*-butyl amide moiety (**14**–**17**) were inactive. Methylenedioxyphenyl compounds were well known to inhibit CYP reaction because they form stable complexes with CYP enzymes;[16] however, inhibitory activity of **12**–**16** were very weak. On the other hand, bisalkaloids (**1**–**5**) and (−)-hinokinin (**21**), containing two methylenedioxyphenyl

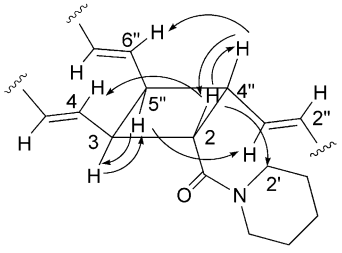

Figure 2. NOE correlations observed for dipiperamide D (**4**).

Table 2. NMR spectral data[a] for dipiperamide E (**5**) in $CDCl_3$

	δ_H	*J* (Hz)	δ_C		δ_H	*J* (Hz)	δ_C
1			165.2 s	2′	3.35	m	42.5 t
2	6.16	dd, 15.1, 1.5	121.2 d		3.45	m	
3	6.90	dd, 15.1, 9.3	144.6 d	3′	1.50 (2H)	m	25.3 t
4	3.61	dq, 1.5, 9.3	46.9 d	4′	1.60 (2H)	m	24.2 t
5	3.78	t, 9.3	46.4 d	5′	1.50 (2H)	m	25.5 t
6			133.3 s	6′	3.42 (2H)	m	44.5 t
7	6.82	d, 2.0	108.5 d	2‴	2.75	m	45.9 t
8			147.9 s		3.07	m	
9			147.0 s	3‴	1.50 (2H)	m	26.0 t
10	6.71	d, 7.8	108.1 d	4‴	1.60 (2H)	m	24.5 t
11	6.73	dd, 7.8, 2.0	121.7 d	5‴	1.50 (2H)	m	26.0 t
1″			169.5 s	6‴	3.40	m	46.8 t
2″	3.50	dd, 9.3, 4.4	46.4 d		3.50	m	
3″	3.97	dt, 4.4, 9.3	41.2 d				
4″	6.29	dd, 15.6, 9.3	127.5 d				
5″	6.43	d, 15.6	131.6 d				
6″			131.7 s				
7″	6.91	d, 2.0	105.7 d				
8″			147.7 s				
9″			146.7 s				
10″	6.72	d, 7.8	108.2 d				
11″	6.78	dd, 7.8, 2.0	120.8 d				
OCH_2O×2	5.94 (4H)	s	100.9 s				
			101.0 s				

[a]Data for piperidine rings (2′-6′ and 2″-6″) may be interchangeable.

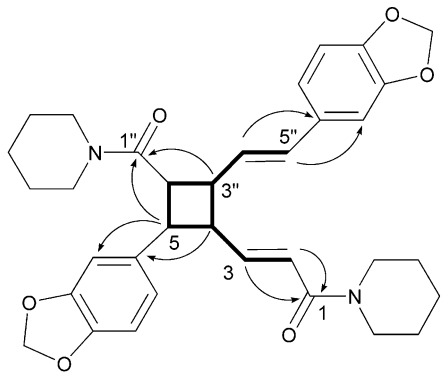

Figure 3. COSY (bold lines) and selected HMBC (arrows) correlations observed for dipiperamide E (**5**).

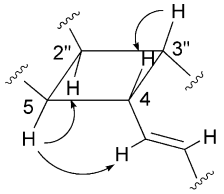

Figure. 4. NOE correlations observed for dipiperamide E (**5**).

rings in the molecules, showed potent CYP inhibition. In 2000 Singh et al. reported the structure–activity relationship (SAR) of piperine and 38 synthetic analogues and found that saturation of the side chain resulted in enhancement of CYP inhibition.[17] Combining our results, the size of the molecule could be one of the reasons. The length of the olefinic chain, however, did not show a clear relationship with the activity. Pyrrolidine ring implied moderate contribution in the case of compounds having acylamide function. In medication, the administration of a potent CYP inhibitor with expensive drugs could lead to cost-savings for patients. Therefore, the study of CYP inhibitors may result in developing alternatives which will reduce the drug dose.

Experimental

General

UV spectra were measured on a Shimadzu UV-1600 UV–visible spectrophotometer. IR spectra were recorded on a Shimadzu IR-460 infrared spectrophotometer. Optical rotations were determined with a Horiba SEPA-300 high sensitive polarimeter. NMR spectra were recorded on a Jeol GSX500 or a Bruker Avance 600 NMR spectrometer in $CDCl_3$ or pyridine-d_5. All chemical shifts were reported with respect to $CDCl_3$ (δ_H 7.26, δ_C 77.0) or pyridine-d_5 (δ_H 7.18). Mass spectra were measured on a Jeol SX-102 or a Jeol GCmate mass spectrometer. Expressed human CYP3A4 was purchased from Gentest Corporation.

Extraction and isolation

White pepper (*Piper nigrum* L.) used in this study was cultivated in Malaysia and was a gift from a Japanese spice company, House Foods Corporation. The pepper (1.0 kg) was refluxed in EtOAc (1.2 L) for 1 h, and then in acetone (1.2 L) for 1 h twice. The combined extracts (65.7 g) were subjected to silica gel chromatography eluting with EtOAc/MeOH (1:1) and then with hexane/acetone (2:1) and ODS chromatography eluting with 70% MeOH/H_2O, followed by reverse-phase HPLC with 75% MeOH/H_2O to afford dipiperamide D (**4**, 4.5 mg, 4.5×10^{-4}%). From this extract, dipiperamides A (**1**, 10.4 mg, 1.0×10^{-3}%), B (**2**, 16.0 mg, 1.6×10^{-3}%), and C (**3**, 4.5 mg, 4.5×10^{-4}%), piperylin (**9**, 3.0 g, 0.3%), piperolein-B (**10**, 15.0 mg, 1.5×10^{-3}%), piperolein-A (**11**, 8.5 mg, 8.5×10^{-4}%), piperine (**12**, 33.0 g, 3.3%), *N-trans*-cinnamoylpiperidine (**13**, 8.5 mg, 8.5×10^{-4}%), guineensine (**14**, 20.0 mg, 2.0×10^{-3}%), pipercide (**15**, 12.0 mg, 1.2×10^{-3}%), retrofractamide A (**16**, 2.0 mg, 2.0×10^{-4}%), (2*E*,4*E*)-*N*-isobutyldecadienamide (**17**, 9.0 mg, 9.0×10^{-4}%), 2,4-decadienoylpiperidine (**18**, 4.4 mg, 4.4×10^{-4}%), and (−)-hinokinin (**21**, 4.6 mg, 4.6×10^{-4}%) were isolated. The pepper (5.0 kg) was separately extracted with acetone at room temperature, and the extract (80 g) was subjected to silica gel chromatography eluting with hexane/EtOAc (1:1) to afford a fraction (3.3 g). A portion (1.5 g) of the fraction was purified by silica gel chromatography eluting with hexane/acetone (2:1) and ODS chromatography eluting with 70% MeOH/H_2O to afford an oily fraction (9.9 mg), to which MeOH (0.5 mL) was added to yield dipiperamide E (**5**, 3.5 mg, 1.5×10^{-4}%) as a white precipitate. From this extract, brachyamide A (**6**, 12.0 mg, 5.1×10^{-4}%), piperamide-C9:1(8*E*) (**7**, 12.2 mg, 5.2×10^{-4}%), piperamide-C9:3(2*E*,4*E*,8*E*) (**8**, 27.5 mg, 1.2×10^{-3}%), *N-trans*-feruloylmethoxytyramine (**19**, 19.0 mg, 8.1×10^{-4}%), and *N-trans*-feruloyltyramine (**20**, 88.0 mg, 3.8×10^{-3}%) were isolated.

Dipiperamide D (4). $[\alpha]_D^{25}$ 0° (*c* 0.077, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 209.0 (4.6), 266.0 nm (4.3); IR ($CHCl_3$) ν_{max} 2930, 1610, 1440, 1250, 1310, 960, 930 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$) see Table 1. HMBC cross peaks ($CDCl_3$): H-2/C-1, C-3, C-4, C-3″, C-4″, C-5″; H-3/C-1, C-2, C-4, C-5, C-4″, C-5″, C-6″; H-4/C-2, C-3, C-5, C-6, C-5″; H-5/C-3, C-6, C-7, C-11; H-7/C-5, C-8, C-9, C-11; H-10/C-6, C-8; H-11/C-5, C-7, C-9; H-2″/C-1″, C-4″; H-3″/C-2, C-1″, C-4″, C-5″; H-4″/C-1, C-2″, C-3″, C-5″; H-5″/C-2, C-4, C-3″, C-4″, C-6″, C-7″; H-6″/C-3, C-4″, C-5″, C-7″, C-8″; H-7″/C-5″, C-6″, C-8″, C-9″, C-13″; H-9″/C-7″, C-10″, C-11″, C-13″; H-12″/C-8″, C-10″; H-13″/C-7″, C-9″, C-11″. ^1H NMR (pyridine-d_5) δ 3.31 (2H, br s), 3.45 (1H, m), 3.46 (1H, m), 3.47 (1H, q, $J=7.8$ Hz, H-5″), 3.55 (1H, m), 3.58 (1H, m), 3.63 (1H, dt, $J=7.8$, 8.3 Hz, H-4″), 3.65 (2H, m), 3.89 (1H, dd, $J=8.3$, 7.8 Hz, H-2), 4.32 (1H, q, $J=7.8$ Hz, H-3), 5.86 (2H, s, OCH_2O), 5.88 (2H, s, OCH_2O), 6.48 (1H, d, $J=15.6$ Hz, H-7″), 6.57 (1H, dd, $J=16.1$, 7.8 Hz, H-4), 6.62 (1H, dd, $J=15.6$, 7.8 Hz, H-6″), 6.66 (1H, d, $J=16.1$ Hz, H-5), 6.71 (1H, d, $J=15.1$ Hz, H-2″), 6.77 (1H, d, $J=8.3$ Hz, H-12″), † 6.78 (1H, d, $J=8.3$ Hz, H-10), † 6.88 (1H, dd, $J=8.3$, 1.5 Hz, H-13″), † 6.89 (1H, $J=8.3$, 1.5 Hz, H-11), † 7.11 (1H, d, $J=1.5$ Hz, H-9″), † 7.13 (1H, d, $J=1.5$ Hz, H-7), † 7.41 (1H, dd, $J=15.1$, 8.3 Hz, H-3″). † May be interchangeable. EIMS (%) *m*/*z* 596 (M^+, 40%), 511 (7), 484 (22), 311 (100),

Table 3. CYP3A4 inhibition of pepper metabolites

Compd	IC_{50} (μM)	Compd	IC_{50} (μM)	Compd	IC_{50} (μM)
1	0.18	**8**	4.2	**15**	123
2	0.45	**9**	3.6	**16**	15
3	0.48	**10**	1.4	**17**	21
4	0.79	**11**	7.6	**18**	38
5	0.63	**12**	17	**19**	28
6	4.9	**13**	65	**20**	26
7	2.8	**14**	14	**21**	2.3
Ketoconazol[a]	0.11				

[a]A typical CYP3A4 inhibitor.[4]

285 (86), 226 (42), 201 (92). HREIMS *m*/*z* 596.2896 ($C_{36}H_{40}N_2O_6$, Δ +1.0 mmu).

Dipiperamide E (5). $[\alpha]_D^{25}$ 0° (*c* 0.18, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 206.5 (3.8), 242.0 (4.1, sh), 266.5 (4.0, sh), 289.0 nm (4.2); IR ($CHCl_3$) ν_{max} 2930, 1609, 1496, 1439, 1038 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$) see Table 2. HMBC cross peaks: H-2/C-1, C-4; H-3/C-1, C-2, C-5; H-4/C-2, C-3, C-6, C-3″, C-4″; H-5/C-3, C-4, C-7, C-11, C-1″; H-7/C-8, C-9; H-10/C-6; H-11/C-7, C-9; H-3″/C-1″; H-4″/C-5″, C-6″; H-5″/C-3″, C-4″, C-7″, C-11″; H-7″/C-6″, C-8″, C-9″, C-11″; H-10″/C-6″, C-8″; H-11″/C-9″. EIMS (%) *m*/*z* 570 (M^+, 7%), 458 (5), 285 (100), 201 (88); HREIMS *m*/*z* 570.2725 ($C_{34}H_{38}N_2O_6$, Δ −0.5 mmu).

Assay of CYP inhibition

CYP activity was based on nifedipine oxidation.[4] Expressed human CYP3A4 (Gentest Corporation, Woburn, USA) was used. The detailed procedure was described in previous papers.[5,6]

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