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Novel dimeric amide alkaloids from *Piper chaba* Hunter: isolation, cytotoxic activity, and their biomimetic synthesis

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ABSTRACT

Chromatographic fractionation of methanol extract from roots of the *Piper chaba* Hunter resulted in the isolation of four new dimeric alkaloids, chabamide H (**1**), I (**2**), J (**3**), K (**4**) together with 11 known compounds (**5**–**15**). Their chemical structures and relative stereochemistry were determined on the basis of the comprehensive spectroscopic techniques (IR, Mass, and NMR) and further confirmed by comparison of the data with those reported in literature. In addition, cytotoxic activities of all the dimeric amides (**1**–**7**) along with their monomers (**8**–**10**) were evaluated against cervical (HELA), breast (MCF-7), liver (HEPG2), colon (HT-29), and colon (COLO-205) cancer cell lines. Among the tested isolates, **5** and **7** exhibited potent cytotoxic activity against COLO-205 cell line with IC_{50} value of 3.10 μg/mL and 0.018 μg/mL, respectively. To prove biogenesis of the newly isolated compounds, biomimetic synthesis has also been carried out via Diels–Alder reaction by using copper(II) salts in aqueous medium.

1. Introduction

The *Piper* species have been used in traditional medicinal systems for thousands of years, including the Chinese and Indian systems, as well as in folklore medicines of Latin America and West Indies.[1] For nearly five decades, investigations into the natural products chemistry of the genus *Piper* have yielded a plethora of amide derivatives of diverse molecular architectures that endow them with remarkable biological activities.[2] The tropical and subtropical species, *Piper nigrum* and *Piper chaba* are particularly noteworthy as they alone appear even more perplexing structural variants.[2,3] The chemical investigation of these species has led to identification of important structural and pharmacological importance, which in turn have spurred novel synthetic methodology and inspired many chemists and biochemists to speculate on the plausible biogenetic relationships among these diverse skeletal families of piperamide alkaloids.[4] Recently, the groups of Tsukamoto et al.[5] Rukachaisirikul et al.,[6] and Wei et al.[7] have highlighted the ever-increasing number of dimeric alkaloid skeletal classes produced by these *Piper* species.

During the past 10 years, great deal of our efforts has been devoted to the phytochemical investigations of the medicinal plants of the genus *Piper* of Piperaceae family, which has resulted in a series of structurally intriguing alkaloids with potential biological activities.[3b,8] In our continuing effort to find bioactive alkaloids from *P. chaba*, a traditional Indian medicinal plant, we have isolated recently two dimeric alkaloids.[9] In order to isolate minor components, we increased the amount of plant material and isolated four new dimeric alkaloids (**1**–**4**) along with 11 known compounds (**5**–**15**) (Fig. 1). These new compounds have been trivially named as chabamide H (**1**), chabamide I (**2**), chabamide J (**3**), and chabamide K (**4**), featuring a six-membered cyclohexene central ring system. Their molecular structures were established by analysis of 1D and 2D NMR, IR, UV, and high-resolution mass spectral data. Furthermore, cytotoxic activities of dimeric alkaloids along with their monomers were screened against HELA, MCF-7, HEPG2, HT-29, and COLO-205 cell lines. Among the tested compounds, chabamide G (**7**) and chabamide (**5**) showed potent activity against the COLO-205 cell line. Herein, we report isolation, structure elucidation, biological activity, and synthesis of the new compounds.

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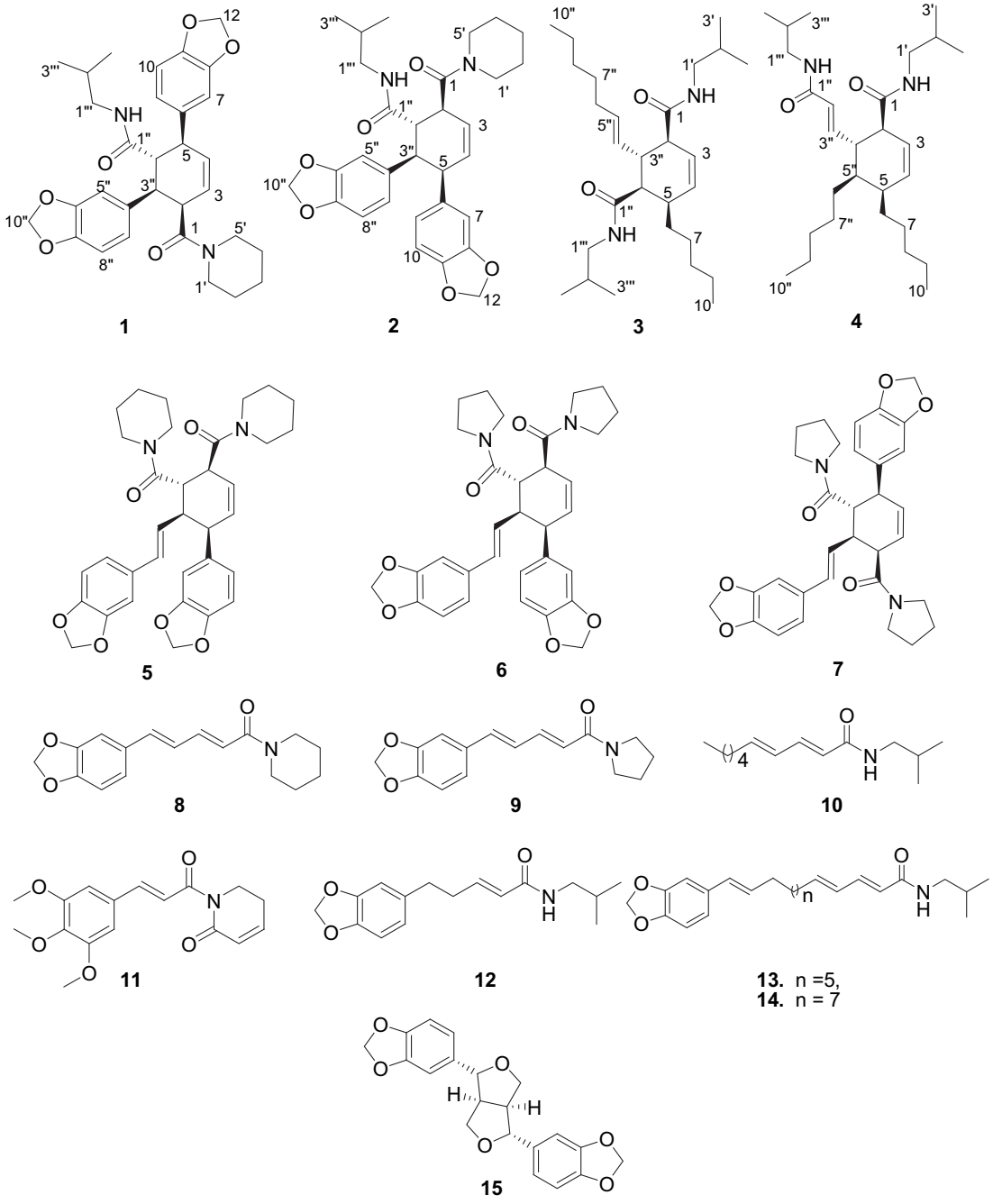


Fig. 1. Compounds isolated from *P. chaba* roots.

2. Results and discussion

The concentrated methanol extract of *P. chaba* was chromatographed on silica gel and the resultant fractions were subjected to bioassay for cytotoxic activity against cancer cell lines. Repeated column chromatography of the bioactive fractions resulted in the isolation of the 15 compounds. Structures of the new compounds (**1**–**4**) were established using IR, MS, 1D, and 2D NMR (HSQC, HMBC, COSY, and NOESY) spectroscopic techniques. The known compounds were identified as chabamide[6] (**5**), chabamide F[9] (**6**), chabamide G[9] (**7**), piperine [3f] (**8**), trichostachine[10] (**9**), pellitorine [3f] (**10**), piplartine[11] (**11**), 4,5-dihydropiperlongumine [3f] (**12**), guineensine [3f] (**13**), brachystamide B [3f] (**14**), and sesamin[12] (**15**) from ^{1}H and ^{13}C NMR data, which were compared with those reported in literature.

2.1. Structural elucidation of chabamide H, I, J, and K (1–4)

Compound **1** was obtained as pale yellow liquid. The UV spectrum showed absorption maxima at λ_{max} (MeOH) 202, 236, and 288 nm, which are typical of dimeric alkaloids.[2] The molecular formula of **1** was established as $C_{31}H_{36}N_2O_6$ by HRESIMS, which provided a molecular ion peak at *m/z* 533.2643 $[M+H]^+$. The IR absorptions implied the presence of tertiary amide (1650 cm^{-1}), NH (3054 cm^{-1}), and aromatic (3050, 1443 cm^{-1}) moiety. The ^{1}H NMR spectrum of **1** ($CDCl_3$) revealed two signals at δ 5.69 (ddd, *J*=9.6, 5.0, 2.6 Hz) and 5.90 (dt, *J*=9.6, 1.5 Hz), which were assigned to *cis*-olefinic protons considering the coupling constant of 9.6 Hz. A pair of aromatic protons at δ 6.97 (1H, d, *J*=1.5 Hz), 6.83 (1H, dd, *J*=8.0, 1.5 Hz), 6.74 (1H, d, *J*=8.0 Hz) and 6.70 (1H, d, *J*=1.3 Hz), 6.68 (1H, d, *J*=8.1 Hz), 6.64 (1H, dd, *J*=8.1, 1.3 Hz) suggested the presence of two

1,3,4-trisubstituted aromatic rings (Tables 1 and 2). It also displayed two methylenedioxy groups at δ 5.91 (2H, br s), 5.86 (2H, br s). In addition, there were characteristic signals of isobutylamide side chain at δ 2.80 (2H, m), 1.30 (1H, m) and two methyls at δ 0.56 (3H, d, J=6.6 Hz), 0.49 (3H, d, J=6.6 Hz), and 5.05 (NH, br t, J=5.8 Hz). ^{13}C NMR spectrum of **1** (Table 2), together with the information from a DEPT spectrum, showed the presence of 31 carbon signals assigned to two methyls, eight methylenes, 13 methines, and eight quaternary carbons including two carbonyl groups. Based on the data mentioned above and comparison of the data with those of nigramide A,[7] the existence of two methylenedioxy phenyl groups led the skeleton of **1** as a dimeric alkaloidal framework.

(δ 46.70) and C-3″ (δ 45.18). Further, the presence of the piperidine ring and isobutyl group at C-2, C-2″ could also be inferred through HMBC correlations observed for H2-1′ to C-1 (δ 170.83) and H2-1‴ to C-1″ (δ 173.15).

The relative configuration of four stereogenic centers in **1** and confirmation of overall structure were achieved by the interpretation of the NOESY spectral data (Fig. 2) and by analysis of ^{1}H NMR coupling constants. The large vicinal coupling constants of H-2″/H-3″ (10.0 Hz) and H-2″/H-5 (10.0 Hz) indicated *anti* relations of H-2″/H-3″ and H-2″/H-5 and the axial orientations for these protons. The small coupling constants observed for H-2/H-3″ (5.5 Hz) indicated *syn* relations of H-2/H-3″. In the NOESY spec-

Table 1
^{1}H NMR data of compounds **1**–**4** in $CDCl_3$ (300 MHz, δ in ppm, mult., J in Hz)

Proton	Chabamide H (**1**)	Chabamide I (**2**)	Chabamide J (**3**)	Chabamide K (**4**)
2	3.78 (m)	3.95 (dq, 10.0, 2.0)	2.80 (m)	2.42 (dq, 10.0, 2.2)
3	5.69 (ddd, 9.6, 5.0, 2.6)	5.62 (dt, 9.8, 1.8)	5.61 (ddd, 10.0, 3.3, 1.8)	5.68 (ddd, 10.0, 3.5, 2.2)
4	5.90 (dt, 9.6, 1.5)	5.90 (ddd, 9.8, 4.7, 1.8)	5.91 (dt, 10.0, 1.8)	5.91 (dt, 10.0, 1.3)
5	3.71 (dq, 10.0, 2.2)	3.60 (m)	2.51 (m)	1.96 (m)
6	—	—	1.80 (m)	1.60 (m)
7	6.97 (d, 1.5)	6.35 (br s)	1.41(m)	1.20 (m)
8	—	—	1.23 (m)	1.23 (m)
9	—	—	1.29 (m)	1.30 (m)
10	6.74 (d, 8.0)	6.56 (d, 7.9)	0.85 (t, 6.8)	0.85 (t, 6.9)
11	6.83 (dd, 8.0, 1.5)	6.32 (dd, 7.9, 1.3)	—	—
12	5.91 (br s)	5.83 (d, 1.3)	—	—
		5.82 (d, 1.3)		
1′	3.55 (m)	3.60 (m)	3.16 (m)	3.07(m)
			3.17 (m)	3.17 (m)
2′	0.69 (m)	1.61 (m)	1.80 (m)	1.75 (m)
3′	1.44 (m)	1.70 (m)	0.93 (d, 6.9)	0.89 (d, 6.2)
			0.91 (d, 6.9)	0.90 (d, 6.2)
4′	1.33 (m)	1.55 (m)	—	—
5′	3.37 (m)	3.61 (m)	—	—
	3.55 (m)			
NH	—	—	5.72 (br t, 5.4)	5.44 (br t, 5.8)
2″	3.48 (t, 10.0)	3.45 (t, 10.0)	2.70 (dd, 10.1, 5.5)	5.74 (d, 15.1)
3″	3.42 (dd, 10.0, 5.5)	3.50 (dd, 10.0, 5.5)	2.58 (q, 10.1)	5.10 (dd, 15.1, 10.0)
4″	—	—	5.25 (dd, 15.1, 10.1)	2.38 (q, 10.0)
5″	6.70 (d, 1.3)	6.10 (br s)	5.50 (m)	2.32 (m)
6″	—	—	1.90 (m)	1.85 (m)
7″	—	—	1.31 (m)	1.30 (m)
8″	6.68 (d, 8.1)	6.45 (d, 8.1)	1.29 (m)	1.23 (m)
9″	6.64 (dd, 8.1, 1.3)	6.06 (br s)	1.26 (m)	1.28 (m)
10″	5.86 (br s)	5.76 (br s)	0.86 (t, 6.8)	0.85 (t, 6.8)
1‴	2.80 (m)	2.71 (m)	2.95 (m)	3.04 (m)
			2.98 (m)	3.17 (m)
2‴	1.30 (m)	1.35 (m)	1.75 (m)	1.78 (m)
3‴	0.56 (d, 6.6)	0.62 (d, 6.0)	0.88 (d, 6.9)	0.92 (d, 6.6)
	0.49 (d, 6.6)	0.59 (d, 6.0)	0.86 (d, 6.9)	0.94 (d, 6.6)
NH	5.05 (t, 5.8)	—	5.60 (br t, 6.0)	5.50 (br t, 5.5)

Analysis of the 2D NMR spectra (HSQC, HMBC, and COSY) of **1** assisted in the determination of its carbon skeleton (Tables 1 and 2, Fig. 2). The sequential proton spin systems detected from the ^{1}H–^{1}H COSY spectrum, H-2/H-3/H-4/H-5 and H-2″/H-3″ [δ 3.78/5.69/5.90/3.71/3.48/3.42] strongly suggested that compound **1** possessed cyclohexene ring similar to those of chabamide F and G.[9] In the HMBC spectrum, the long-range correlations of two methylene protons at δ 5.91 with 146.30 (C-9), 147.70 (C-8), at δ 5.86 with δ 147.60 (C-6″) and δ 146.30 (C-7″) suggested that the two methylenedioxy rings were fused to C-8, C-9, and C-6″, C-7″ of the two aromatic rings, respectively. The locations of the two carbonyl groups at C-1 and C-1″ were based on the correlations for δ 3.78 (H-2) to δ 170.83 (C-1) and δ 3.48 (H-2″) to δ 173.15 (C-1″). Concerning the connections of the two phenyl rings, HMBC spectrum exhibited correlations of H-7 (δ 6.97, d, J=1.5 Hz), H-11 (6.83, dd, J=8.0, 1.5 Hz)/C-5 (δ 46.70), H-5 (3.71, dq, J=10.0, 2.2 Hz)/C-6 (137.64), H-3″ (3.42, dd, J=10.0, 5.5 Hz)/C-4″ (δ 135.62), which clearly indicated that these rings were bonded to the cyclohexene ring at C-5

trum, the correlations between H-3″/H-5 and the absence of NOE effects between H-2″/H-5 and H-2″/H-3″ supported the above result. These data indicated a β-orientation for H-2″ and α-orientation for H-3″, H-5, and H-2.

Further evidence regarding the chemical structure of **1** was accomplished by its mass spectrum (EIMS), showing intense peak at m/z 532 (100%). The fragmentation pattern in the mass spectrum (EIMS) showing the base peak at m/z 285 (30%) corresponds to the piperine and remaining m/z 247 (5%) corresponded to the fagaramide, suggesting these two units were joined together by the Diels–Alder reaction (Fig. 3). On the basis of these spectral data, the structure of **1** was unambiguously established and trivially named as chabamide H.

Compound **2** was isolated as a yellow oil, had the same molecular formula $C_{31}H_{36}N_2O_6$ as that of the **1** on the basis of the HRESIMS analysis (m/z 533.2647 $[M+H]^+$). The close resemblance of ^{1}H NMR and ^{13}C NMR (Tables 1 and 2) data for **2** indicated that **2** was a stereoisomer of the compound **1**. The only distinct difference

Table 2
^{13}C NMR data of compounds **1**–**4** in $CDCl_3$ (75 MHz, δ in ppm)

No.	Chabamide H (**1**)	Chabamide I (**2**)	Chabamide J (**3**)	Chabamide K (**4**)
1	170.83	170.80	174.40	171.67
2	41.51	44.35	49.06	45.31
3	124.43	123.92	122.80	124.66
4	133.44	131.78	134.05	133.62
5	46.70	47.07	37.95	40.51
6	137.64	132.02	33.70	33.60
7	109.06	110.07	29.71	26.65
8	147.70	146.98	31.93	31.92
9	146.30	146.86	28.30	22.71
10	108.16	107.52	14.10	14.07
11	121.68	123.75	—	—
12	100.82	100.72	—	—
1′	47.03	46.92	46.99	46.90
2′	25.75	26.75	28.50	28.55
3′	24.27	24.54	20.18	20.11
4′	25.24	25.69	—	—
5′	43.04	43.43	—	—
1″	173.15	173.47	172.30	162.44
2″	51.29	45.46	48.60	125.31
3″	45.18	42.91	43.03	147.19
4″	135.62	135.02	128.8	44.44
5″	108.81	109.08	133.95	40.05
6″	147.60	146.24	32.50	32.89
7″	146.30	145.72	29.03	27.45
8″	108.25	107.43	31.41	31.40
9″	121.35	121.44	22.72	22.53
10″	100.82	100.52	14.04	14.05
1‴	46.36	46.48	46.88	46.82
2‴	28.14	28.41	28.37	28.40
3‴	19.72	19.88	20.08	20.11
	19.58	19.84	20.08	20.11

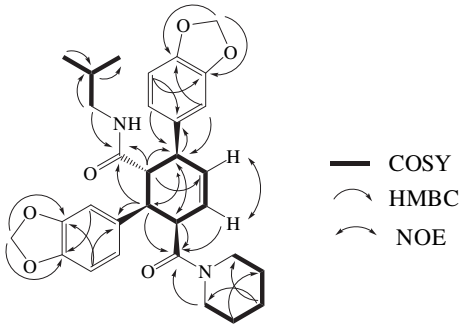


Fig. 2. COSY, NOESY, and important HMBC correlations of **1**.

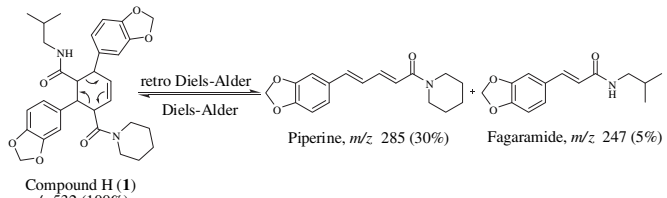


Fig. 3. Mass fragmentation of compound **1**.

between the two compounds was that the *ortho* orientation of the two carbonyl groups in **2**, whereas in **1** they were *meta* orientated. As in **1**, major portion of **2** was assembled by the interpretation of COSY, HSQC, and HMBC spectral data (Fig. 4).

The COSY spectrum of **2** revealed the presence of a cyclohexene ring (−C-2−C-3−C-4−C-5−C-3″−C-2″−). The location of the carbonyl groups at C-2 and C-2″ was based on the correlations for δ 5.62 (H-3), 3.95 (H-2), and 3.45 (H-2″) to δ 170.80 (C-1) and δ 3.50 (H-3″) and 3.95 (H-2) to δ 173.47 (C-1″) as observed in

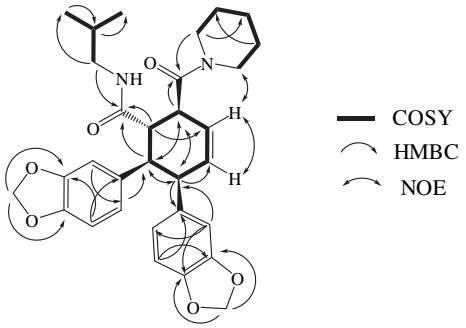


Fig. 4. COSY, NOESY, and important HMBC correlations of **2**.

HMBC spectrum. The relative configurations of the stereogenic centers of **2** were elucidated on the basis of the $^1H-^1H$ coupling constants and NOESY correlations as shown in Fig. 4. The large coupling constants for H-2/H-2″ (10.0 Hz) and H-2″/H-3″ (10.0 Hz) indicated *anti* relations of H-2/H-2″ and H-2″/H-3″ and the axial orientations for these protons. The small coupling constants observed for H-3″/H-5 (5.5 Hz) indicated *syn* relations of H-3″/H-5. In the NOESY spectrum, the correlations between H-3″/H-2 and the absence of NOE effects between H-2″/H-2 and H-2″/H-3″ supported the above result. These data indicated a β-orientation for H-2″ and α-orientation for H-2, H-3″, and H-5. It is suggested that **2** had the same relative stereochemistry as that of **1**. Based on these data the stereostructure of **2** was established and trivially named as chabamide I.

Compound **3** was isolated as a pale yellow oil, had the molecular formula of $C_{28}H_{50}N_2O_2$, as deduced from the HRESIMS *m*/*z* 447.3955 $[M+H]^+$. IR spectrum implied the presence of carbonyl (1642 cm^{-1}) and NH (3228 cm^{-1}). The ^1H NMR spectrum of **3** revealed the presence of a trans double bond at δ 5.25 (dd, *J*=15.1, 10.1 Hz, H-4″), 5.50 (m, H-5″), two isobutylamide groups at δ 3.16 (m, H1-1′), 3.17 (m, H2-1′), 1.80 (m, H-2′), 0.93 (d, *J*=6.9 Hz, H-3′), 0.91 (d, *J*=6.9 Hz, H-3′), 5.72 (br t, *J*=5.4 Hz, NH) and δ 2.95 (m, H1-1‴), 2.98 (m, H2-1‴), 1.75 (m, H-2‴), 0.88 (d, *J*=6.9 Hz, H-3‴), 0.86 (d, *J*=6.9 Hz, H-3‴), 5.60 (br t, *J*=6.0 Hz, NH), *n*-amyl group, and 1-heptene unit at δ 1.80 (m, H-6), 1.41(m, H-7), 1.23 (m, H-8), 1.29 (m, H-9), 0.85 (t, *J*=6.8 Hz, H-10) and δ 5.25(dd, *J*=15.1, 10.1 Hz, H-4″), 5.50 (m, H-5″), 1.90 (m, H-6″), 1.31 (m, H-7″), 1.29 (m, H-8″), 1.26 (m, H-9″), 0.86 (t, *J*=6.8 Hz, H-10″), respectively (Table 1). The ^{13}C NMR spectrum displayed the presence of 28 carbon atoms (Table 2), and was further classified by DEPT experiments into categories of six methyls, 10 methylenes, 10 methines, and two quaternary carbons including two carbonyls (δ 174.40 and 172.30). The analyses of the ^1H and ^{13}C NMR spectral data of **3** (Tables 1 and 2) showed a high degree of similarity with dimeric alkaloid nigramide O[7] except for the presence of double bond in the aliphatic chain. Furthermore, the detailed elucidation of the 2D NMR data (COSY, HSQC, HMBC) determined the planar structure of **3**.

The ^1H-homodecoupling NMR experiments of **3** revealed ^1H spin-coupling connectivities H-2 (δ 2.80, ddd, *J*=5.4, 3.5, 1.8 Hz) to H-3 (δ 5.61, ddd, *J*=10.0, 3.3, 1.8 Hz) to H-4 (δ 5.91, dt, *J*=10.0, 1.8 Hz) to H-5 (δ 2.51, m) to H-2″ (δ 2.70, dd, *J*=10.1, 5.5 Hz) to H-3″ (δ 2.58, q, *J*=10.1 Hz) via cyclohexene ring protons. The *meta* orientation of the carbonyl and isobutylamide groups was established by HMBC correlations for δ 2.80 (m, H-2), 5.61 (ddd, *J*=10.0, 3.3, 1.8 Hz, H-3), 2.58 (q, *J*=10.1 Hz, H-3″), 3.17 (m, H-1′)/δ 174.40 (C-1) and δ 2.70 (dd, *J*=10.1, 5.5 Hz, H-2″), 2.51 (m, H-5), 2.58 (q, *J*=10.1 Hz, H-3″), 2.98 (m, H-1‴)/δ 172.30 (C-1″) (Fig. 5). Furthermore, the $^1H-^1H$ COSY cross-peaks between δ 2.58 (q, *J*=10.1 Hz, H-3″) and δ 5.25 (dd, *J*=15.1, 10.1 Hz, H-4″), and δ 5.50 (m, H-5″) and δ 2.51 (m, H-5), 1.80 (m, H-6), 1.41 (m, H-7), coupled with the HMBC correlation for δ 5.50 (m, H-5″) to δ 29.71 (C-7″), δ 1.41 (m, H-7) to δ 37.95 (C-5) established the

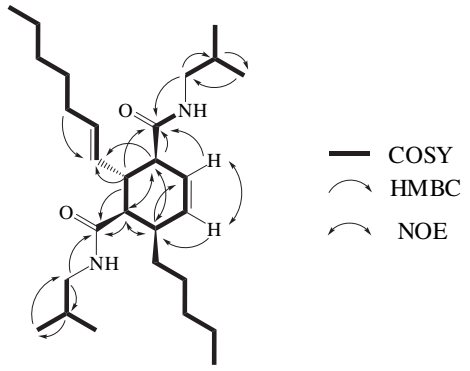


Fig. 5. COSY, NOESY, and important HMBC correlations of **3**.

attachment of the 1-heptene and *n*-amyl groups at C-3″ and C-5, respectively (Fig. 5). The analyses of the ^{1}H–^{1}H coupling constants and NOESY data allowed us to determine the relative stereochemistry of **3**. The large coupling constants of H-3″/H-2 and H-3″/H-2″ (10.1 Hz) indicated *anti* relations and axial orientations for these protons. The small coupling constants observed for H-2″/H-5 (5.5 Hz) indicated *syn* relations of H-2″/H-5. In the NOESY spectrum, correlations between H-2/H-2″ and the absence of NOE cross-peaks between H-3″/H-2 and H-3″/H-2″ supported the above result. These data indicated a β-orientation for H-3″ and α-orientation for H-2, H-2″, and H-5. Thus, based on these spectral data the stereostructure of **3** was confirmed and trivially named as chabamide J.

Compound **4** had the same molecular formula $C_{28}H_{50}N_2O_2$ as that of chabamide J (**3**) deduced from its HRESIMS, *m*/*z* 447.3951 $[M+H]^+$. Its IR spectrum displayed absorptions of carbonyl group (1630 cm^{-1}), NH (3293 cm^{-1}), and double bond (1462 cm^{-1}), respectively. The characteristic IR absorption bands, as well as the ^{1}H and ^{13}C NMR spectra of **3** and **4**, indicated that both had similar structural features, except for double bond conjugated to the carbonyl group in **4** instead of a double bond in aliphatic chain.[7] Appearance of carbonyl group in ^{13}C NMR spectra at δ 162.44 supports the presence of unsaturated α,β-carbonyl group in the molecule.

As to the connections of these double bond protons and carbons, the ^{1}H-homodecoupling experiments of **4** in $CDCl_3$ revealed the ^{1}H spin-coupling connectivities of δ 5.74 (d, *J*=15.1 Hz, H-2″), to δ 5.10 (dd, *J*=15.1, 10.0 Hz, H-3″) and δ 2.38 (q, *J*=10.0 Hz, H-4″). Further, HMBC correlations of **4** (Fig. 6) for δ 5.74 (d, *J*=15.1 Hz, H-2″), 5.10 (dd, *J*=15.1, 10.0 Hz, H-3″), 2.32 (m, H-5″)/δ 44.44 (C-4″) clarified the assignments of C-2″, C-3″, and C-5″, as shown in Table 1. Each of the ^{1}H spin-coupling constants between the side chain (*n*-amyl) protons and protons on the cyclohexene ring in **4** agreed with their respective ones in **3** within experimental error. These results indicated that **4** was an analog of **3** with shift of the double bond in the chromophore of **3**. The trans geometries between H-2″ and H-3″ on the double bond in **4** were determined by the magnitude (15.1 and 10.0 Hz) of their ^{1}H spin-coupling constants. The *ortho* orientation of the two carbonyls with isobutylamide groups was established by HMBC correlations for δ 2.42 (dq, *J*=10.0, 2.2 Hz, H-2), 5.68 (ddd, *J*=10.0, 3.5, 2.2 Hz, H-3), 2.38 (q, *J*=10.0 Hz, H-4″), 3.07 (m, H1-1′)/δ 171.67 (C-1) and δ 5.10 (dd, *J*=15.1, 10.0 Hz, H-3″), 5.74 (d, *J*=15.1 Hz, H-2″), 3.04 (m, H1-1‴)/δ 162.44 (C-1″) (Fig. 6). The analyses of the ^{1}H–^{1}H coupling constants and NOESY data allowed us to determine the relative stereochemistry of **4**. The coupling constants of H-4″/H-2 and H-4″/H-5″ (10.0 Hz) indicated *anti* relations of H-4″/H-2 and H-4″/H-5″ and the axial orientations for these protons. In the NOESY spectrum, correlations between H-2/H-5″ and the absence of NOE effects between H-4″/H-2 and H-4″/H-5″ supported the above result. These data indicated a β-orientation for H-4″ and α-orientation for H-2, H-5″, and H-5. Thus, based on these spectral data the stereostructure of **4** was confirmed and trivially named as chabamide K.

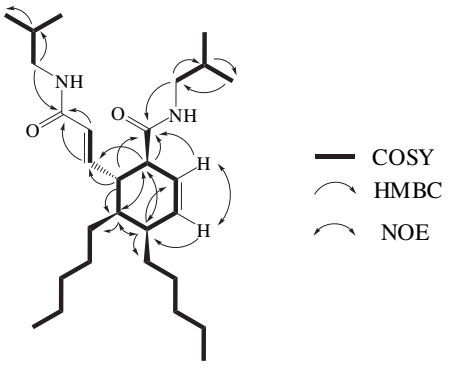
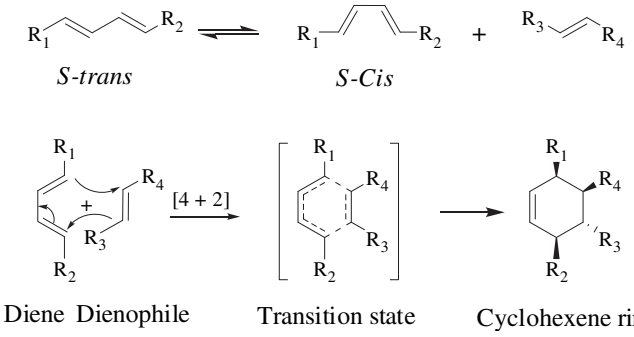


Fig. 6. COSY, NOESY, and important HMBC correlations of **4**.

The dipiperamides are a family of structurally diverse class of metabolites and usually isolated from the *Piper* species.[5,6] As postulated by Wei et al.[7] these compounds are presumably generated by intermolecular Diels–Alder reaction of the same or different two monomeric alkaloids to afford a fused cyclic (cyclohexene) system. Compounds **1**–**4** are novel cyclohexene family of dimeric alkaloids, which are not reported till date. From the biosynthetic view point, chabamides H-I, may be generated by the intermolecular Diels–Alder reaction between same or different alkamides. Further, zero optical rotation of all the new compounds (**1**–**4**) suggested that these are racemates (Scheme 1).[7]



Scheme 1. Plausible biosynthetic route for the formation of cyclohexane ring.

3. Cytotoxic studies

As a primary screen for cytotoxic activity, cancer cell growth inhibitory properties of chabamides **1**–**7** along with their monomeric amides **8**–**10** were examined using cervical cancer (HELA), breast cancer (MCF-7), liver cancer (HEPG2), and colon cancer (HT-29), (COLO-205) cell lines by MTT assay.[13]

As shown in Table 3, all dimeric alkaloids displayed potent activity than the corresponding monomeric amides in the tested cell lines. In particular, chabamide G (**7**) showed significant cytotoxic activity (IC_{50} 0.018 μg/mL) against the COLO-205 cancer cell line. However, its monomeric amide, trichostachine is not active against tested cell lines. Similarly, chabamide (**5**) exhibited potent activity than its monomer against HELA, MCF-7, HEPG2, HT-29, and COLO-205 cell lines. To the best of our knowledge this is the first report of the isolation and structural characterization of four new dimeric alkaloids and their biological activities. This study not only expands the number of discovered dimeric alkaloids from *P. chaba* but also explores the structure–activity relationship for cytotoxic activity and suggests possibilities for the design and synthesis of cytotoxic drugs.

Table 3
Cytotoxic effects (IC_{50} μg/mL) of the chabamides **1**–**7** along with their corresponding monomeric amides **8**–**10**

Extract/dimer/monomer		HELA (cervical)	MCF-7 (breast)	HEPG2 (liver)	HT-29 (colon)	COLO-205 (colon)
IC_{50} (μg/ml)	MeOH extract	35.7±6.71	26.3±2.17	6.46±0.39	NA	102.38±1.48
	Chabamide H (**1**)	NA	170.1±6.60	135.0±0.76	NA	37.0±0.96
	Chabamide I (**2**)	140.3±29.4	NA	NA	NA	42.9±0.15
	Chabamide J (**3**)	NA	NA	NA	201.2±4.47	NA
	Chabamide K (**4**)	85.3±18.2	NA	195.3±13.3	177.7±1.16	169.5±1.86
	Chabamide (**5**)	108.3±21.5	22.3±3.88	34.7±1.30	NA	3.10±4.22
	Chabamide F (**6**)	64.8±14.5	27.1±4.19	24.2±0.92	140.9±4.56	98.63±2.44
	Chabamide G (**7**)	46.3±7.0	27.9±0.78	58.6±2.0	NA	0.018±0.012
	Piperine (**8**)	171.8±28.6	NA	101.7±4.97	NA	5.89±1.30
	Trichostachine (**9**)	NA	NA	NA	NA	NA
	Pellitorine (**10**)	103.4±6.28	133.08±1.68	15.08±1.78	NA	11.6±0.65

NA-Not active.

Table 4
Optimization of solvent, catalyst, and temperature for Diels–Alder reaction of piperine (**8**) with fagaramide (**16**)

Entry	Solvent	Catalyst/ligand	Catalyst (mol %)	Temp (°C)	Overall yield[a] (%)
1	Toluene	$FeSO_4·7H_2O$	20	110	5
2	Toluene	$CoCl_2·6H_2O$	10	110	7
3	Toluene	$CuSO_4·5H_2O$	10	110	10
4	Toluene	$CuCl_2$	10	110	15
5	Toluene	$Cu(NO_3)_2·2.5H_2O$	10	110	20
6	Toluene	$Cu(OAc)_2$	10	110	5
7	Toluene	$ZnCl_2$	10	110	8
8	Toluene	$Zn(OAc)_2$	10	110	12
9	Toluene	$PdCl_2$	10	110	10
10	Toluene	$Pd(OAc)_2$	10	110	15
11	Toluene	$Pd(PPh_3)_4$	10	110	10
12	Xylene	$CoCl_2·6H_2O$	10	135	10
13	Xylene	$CuSO_4·5H_2O$	10	135	15
14	Xylene	$CuCl_2$	10	135	40
15	Xylene	$Cu(NO_3)_2·2.5H_2O$	10	135	65
16	Xylene	$Cu(NO_3)_2·2.5H_2O$	15	135	65
17	Xylene	$PdCl_2$	10	135	35
18	Xylene	$Pd(OAc)_2$	10	135	40
19	Xylene	$Pd(PPh_3)_4$	10	135	35
20	Water	$CoCl_2·6H_2O$	10	100	12
21	Water	$CuSO_4·5H_2O$	10	100	15
22	Water	$CuCl_2$	10	100	55
23	Water	$Cu(NO_3)_2·2.5H_2O$	10	100	75
24	Water	$PdCl_2$	10	100	50
25	Water	$Pd(OAc)_2$	10	100	55
26	Water	$Pd(PPh_3)_4$	10	100	25
27	Neat	$CuCl_2$	10	130	47
28	Neat	$Cu(NO_3)_2·2.5H_2O$	10	130	60
29	Neat	$PdCl_2$	10	130	45
30	Neat	$Cu(NO_3)_2·2.5H_2O$	10	130	55
31	Neat	$Pd(OAc)_2$	15	130	50

All reactions were carried out for 70 h.
[a] Combined yield of all adducts after HPLC.

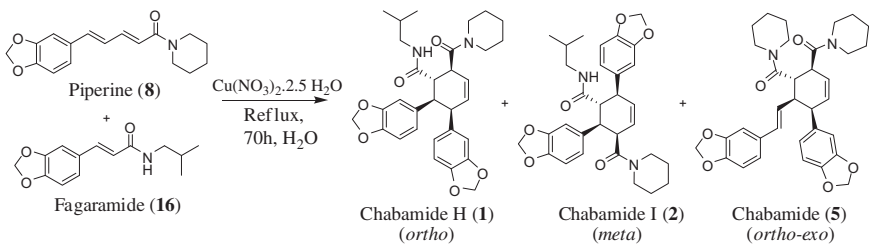
4. Biomimetic synthesis of cyclohexene type of dimeric amide alkaloids (1–4)

On the basis of a biosynthetic hypothesis by the intermolecular Diels–Alder reaction, we chose piperine (**8**), *trans*-fagaramide (**16**), and pellitorine (**10**) substrates to perform the biomimetic synthesis of the chabamides (**1**–**4**). Although it is well known that transition metals accelerate the cycloaddition reactions, finding the suitable reagent and solvent for the reactions is a major challenge for the organic chemists. As a first attempt, we chose piperine (**8**), pellitorine (**10**), isolated from the *P. nigrum* and fagaramide (**16**), and synthesized by the following procedure.[14] These monomers were used to initiate our study of Diels–Alder dimerization reaction in the presence of various transition metal catalysts using different solvents. The results of these reactions are shown in the Table 4, which clearly demonstrates that reactions were not triggered by using $FeSO_4$, $CoCl_2$, $NiCl_2$ in toluene even after heating for long hours. However, the reaction using $Cu(NO_3)_2·2.5H_2O$ in water seems to be interesting as it gave the higher yields (entry 23) of the adducts. To find the optimum catalyst loading, we have performed the reactions with 5–10 mol % ratio of Cu(II) salts and highest catalytic activity was attained using 10 mol % of Cu(II) salts. The role of copper salts in this reaction can be attributed to its Lewis acid ability, which reduced the HOMO–LUMO energy gap between diene and dienofile.

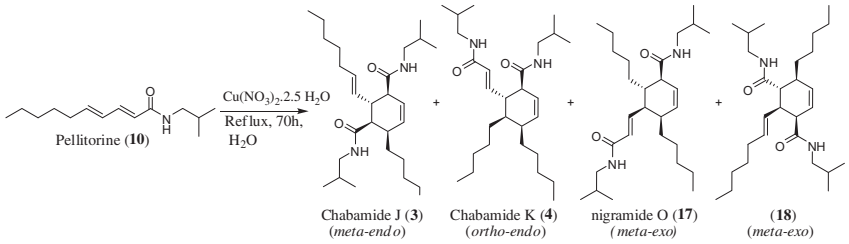
Thus, $Cu(NO_3)_2·2.5H_2O$ catalyzed cycloaddition reactions (Scheme 2) of piperine (**8**) and *trans*-fagaramide (**16**) were carried out using water and xylene as solvents to give resultant cycloadducts **1**, **2**, and **5**. This reaction showed overall yield of 65% in xylene and 75% in water, respectively. It is important to mention that *ortho* products were formed predominantly than *meta* in both solvents. All the products were well characterized by spectroscopic techniques (NMR and Mass).

4.1. Cycloaddition reactions of pellitorine (10)

Using the above standard conditions, we also attempted the synthesis of chabamide J (**3**) and chabamide K (**4**) through the dimerization of pellitorine (**10**) (Scheme 3). The resulted cycloadducts **3**, **4**, **17**, and **18** were characterized by using the NMR, mass spectral data and compared with the isolated compound. Physical and spectral data of adducts **17** were found identical with nigramide O, which was isolated previously from *P. nigrum*.[7] The adduct **18** is a new dimer from pellitorine and its structure was elucidated by 1D and 2D spectral data. Similar to the cycloaddition of fagaramide and piperine, this reaction also showed good overall yield in



Scheme 2. Cycloaddition reactions of piperine (**8**) and fagaramide (**16**).



Scheme 3. Cycloaddition reaction of pellitorine (**10**).

water (70%) than in xylene (60%). However, *meta* products were predominantly formed than the *ortho* products.

5. Conclusion

Four new compounds **1**–**4** along with 11 known compounds were isolated from *P. chaba* Hunter. All the compounds displayed moderate cytotoxicity against human cancer cell lines. Among the tested isolates, **5** and **7** showed potent cytotoxic activity against COLO-205 cell line with IC_{50} value of 3.10 μg/mL and 0.018 μg/mL. We also synthesized the new compounds (**1**–**4**) using the $Cu(NO_3)_2{\cdot}2.5H_2O$ as a catalyst and water as solvent. The synthesis appears to be general, and it should allow access to the preparation of a variety of structural analogs for investigation of pharmacological structure–activity relationships.

6. Experimental

6.1. General experimental procedures

Optical rotations were measured using a JASCO DIP 300 digital polarimeter and using 1 mL cell at 25 °C. IR spectra were recorded on a Nicolet-740 spectrometer in KBr pellets. UV spectra were recorded on Agilent G1315B Diode Array Detector. The NMR spectra were recorded on a Bruker FT-300 MHz spectrometer at 300 MHz for ^1H and 75 MHz for ^{13}C, respectively, using TMS as an internal standard. The chemical shifts are expressed as δ values in parts per million (ppm) and the coupling constants (J) are given in hertz (Hz). HRESIMS analyses were performed on Agilent Technologies 6510 Q-TOF LC–MS. The 2D experiments (^1H–^1H COSY, HSQC, HMBC, NOESY) were performed using standard Bruker micro programs. Column chromatography was performed with silica gel (100–200 mesh, Qingdao Marine Chemical, Inc., Qingdao, China). Preparative HPLC was performed on a Dionex P680 equipped with PDA detector using Zorbax SB C18 (9.4×50 mm, 5 μ) column, analytical TLC was performed on precoated Merck plates (60 F_{254}, 0.2 mm) with the solvent system EtOAc/hexane (50:50), and compounds were viewed under a UV lamp and sprayed with 10% H_2SO_4, followed by heating.

6.2. Plant material

The roots of the plant *P. chaba* were collected from Indian Medicines and Pharmaceuticals Limited (IMPCL), Uttarakhand, India. It was authenticated by Dr. K. Madhava Chetty, and a voucher specimen was deposited in the herbarium of the Botany department, Sri Venkateswara University, Tirupati, Andhra Pradesh, India.

6.3. Extraction and isolation

The roots of *P. chaba* (5 kg) were shade dried, powdered, and extracted with methanol at room temperature for 48 h. The resulting methanol extract was evaporated to dryness under reduced pressure, affording syrupy residue (50 g), which exhibited cytotoxic activity. Active MeOH extract (50 g) was subjected to column chromatography (silica gel, 100–200 mesh, eluting with hexane/EtOAc/acetone/$CHCl_3$/MeOH mixtures of increasing polarity) to give five major fractions (F1–F5). Fraction F1 was purified by repeated flash chromatography on silica gel (100–200 mesh) by eluting with EtOAc/hexane (2:8) to yield 1.25 g of piplartine (**11**). Fraction F2 was subjected to repeated column chromatography on silica gel with the elution of EtOAc/hexane (3:7), which gave 0.7 g of pellitorine (**10**), 0.3 g of piperine (**8**), 0.06 g of trichostachine (**9**), 0.054 g of 4,5-dihydropiperlongumine (**12**), and 0.05 g of guineensine (**13**), respectively. Purification of the fraction F3 was achieved by the flash chromatography with EtOAc/hexane (4:6) to give 0.050 g of brachystamide B (**14**), 0.030 g of sesamin (**15**). Fraction F5 was purified by reverse phase HPLC using Eurospher C18 (60×16 mm, 15 μ) with acetonitrile/water (45:55) to give two sub fractions, of which sub fraction A, was again subjected for purification by reverse phase HPLC using Zorbax SB C18 (9.4×50 mm, 5 μ) with gradient elution, 0–10 min, acetonitrile/water (1:1); 10–35 min, acetonitrile/water (7:3); 36–40 min, acetonitrile/water (1:1) yielded the chabamide (**5**) 0.015 g, chabamide F (**6**) 0.015 g, and chabamide G (**7**) 0.010 g, respectively. Sub fraction B was subjected to reverse phase HPLC fractionation using Phenomenex Luna C18 (250×10 mm, 10 μ) column, solvent system: 80% acetonitrile in water, flow rate: 1.5 mL/min, detection: PDA, retention time: 12.8, 14.1 min] to give pure chabamide H (**1**) 0.009 g, chabamide I (**2**) 0.010 g and another fraction also purified by HPLC using Zorbax SB C18 (9.4×50 mm, 5 μ) column, solvent system: 70% acetonitrile in water, flow rate: 1.5 mL/min, detection: 210 UV, retention time: 5.4, 10.5 min] to give chabamide J (**3**) 0.008 g, chabamide K (**4**) 0.007 g in pure form.

6.4. Chabamide H (1)

Yellow liquid; $[\alpha]_D^{25}$ ±0 (*c* 0.2, $CHCl_3$); IR (KBr): ν_{max} 3054, 2923, 2856, 1634, 1486, 1443, 1242, 1038, 933, and 771 cm^{-1}; ^1H and ^{13}C NMR (see Tables 1 and 2); EIMS *m*/*z* (%): 532 (100%), 459 (25%), 432 (49%), 347 (35%), 319 (71%), 285 (30%), 247 (5%), 201 (22%), 173 (12%), 145 (10%), 135 (52%), 112 (55%), 84 (20%), 69 (30%); HRESIMS *m*/*z*, 533.2643 $[M+H]^+$, calcd for $C_{31}H_{36}N_2O_6$ 533.2646.

6.5. Chabamide I (2)

Yellow liquid; $[\alpha]_D^{25}$ ±0 (*c* 0.2, $CHCl_3$); IR (KBr): ν_{max} 3430, 2928, 2865, 1625, 1487, 1443, 1239, 1038, 933, and 761 cm^{-1}; ^1H and ^{13}C NMR (see Tables 1 and 2); EIMS *m*/*z* (%): 532 (5%), 447 (100%), 432 (5%), 419 (26%), 347 (12%), 319 (15%), 285 (25%), 247 (22%), 231 (60%), 215 (32%), 201 (39%), 175 (26%), 145 (16%), 135 (37%), 115 (46%), 84 (22%), 70 (28%), 57 (42%), 41 (60%); HRESIMS *m*/*z*, 533.2647 $[M+H]^+$, calcd for $C_{31}H_{36}N_2O_6$ 533.2646.

6.6. Chabamide J (3)

Yellow liquid; $[\alpha]_D^{25}$ ±0 (*c* 0.2, $CHCl_3$); IR (KBr): ν_{max} 3228, 3083, 2955, 2923, 2858, 1642, 1549, 1462, 1376, 1268, and 769 cm^{-1}; ^1H

and ^{13}C NMR (see Tables 1 and 2); EIMS m/z (%): 446 (34%), 375 (46%), 346 (48%), 273 (47%), 223 (13%), 203 (12%), 152 (7%), 105 (17%), 91 (34%), 81 (22%), 57 (100%), 41 (78%), 43 (67%); HRESIMS m/z, 447.3955 $[M+H]^+$, calcd for $C_{28}H_{50}N_2O_2$ 447.3945.

6.7. Chabamide K (4)

Yellow liquid; $[\alpha]_D^{25}$ ±0 (*c* 0.2, $CHCl_3$); IR (KBr): ν_{max} 3293, 2922, 2850, 1738, 1630, 1548, 1462, 1373, 1113, and 770 cm^{-1}; ^1H and ^{13}C NMR (see Tables 1 and 2); EIMS m/z (%): 446 (6%), 375 (6%), 346 (8%), 276 (7%), 223 (20%), 201 (7%), 152 (8%), 98 (20%), 85 (23%), 71 (33%), 57 (100%), 43 (62%), 41 (32%); HRESIMS m/z, 447.3951 $[M+H]^+$, calcd for $C_{28}H_{50}N_2O_2$ 447.3945.

6.8. Diels–Alder reaction of piperine (8) and fagaramide (16)

To a stirred mixture of piperine (50.0 mg, 0.175 mmol), fagaramide (43 mg, 0.175 mmol), deionized water (2 mL) in a round bottom flask was added copper(II) nitrate (5 mg, 10 mol %) and refluxed for 70 h in an oil bath. After completion of the reaction, monitored by TLC (dipped in 5% w/v solution of phosphomolybdic acid in methanol and heating), the reaction mixture was cooled to room temperature and diluted with water (3 mL), extracted with EtOAc (2×5 mL), the combined organic layers were dried over anhydrous Na_2SO_4 and concentrated in vacuum. The residue obtained was purified by reverse phase HPLC using Phenomenex Luna C18 (250×10 mm, 10 μ), solvent system: 80% acetonitrile in water, flow rate: 1.5 mL/min, to give pure adducts (**1**) 0.049 g, (**2**) 0.014 g, and (**5**) 0.007 g. The structure of adduct **5** was established by spectroscopic methods (NMR, MS, and IR), whose data is identical with chabamide.[6]

6.9. Diels–Alder reaction of pellitorine (10)

To a stirred mixture of pellitorine (50.0 mg, 0.224 mmol), deionized water (2 mL) in a round bottom flask was added copper (II) nitrate (6 mg, 10 mol %) and refluxed for 70 h in an oil bath. After completion of the reaction, monitored by TLC (dipped in 5% w/v solution of phosphomolybdic acid in methanol and heating), the reaction mixture was cooled to room temperature and diluted with water (3 mL), extracted with EtOAc (2×5 mL), the combined organic layers were dried over anhydrous Na_2SO_4 and concentrated in vacuum. The residue obtained was purified by HPLC using Zorbax SB C18 (9.4×50 mm, 5 μ), solvent system: 70% acetonitrile in water, flow rate: 1.5 mL/min, detection: 210 UV to give pure adducts (**3**) 0.0315 g, (**4**) 0.028 g, (**17**) 0.007 g, and (**18**) 0.0035 g. The spectral data of **17** identical with nigramide O, which is isolated from *P. nigrum*.[7] The spectral data of **18**: Yellow liquid; $[\alpha]_D^{25}$ ±0 (*c* 0.75, $CHCl_3$); IR (KBr): ν_{max} 3304, 2923, 2852, 1648, 1465, 1234, and 1159 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 0.86 (3H, d, *J*=6.7 Hz, H-3‴), 0.86 (3H, t, *J*=6.5 Hz, H-10), 0.87 (3H, d, *J*=6.7 Hz, H-3‴), 0.88 (3H, t, *J*=6.5 Hz, H-10″), 0.90 (3H, d, *J*=6.7 Hz, H-3′a), 0.91 (3H, d, *J*=6.7 Hz, H-3′b), 1.20 (2H, m, H-8), 1.27 (2H, m, H-9″), 1.27 (2H, m, H-9), 1.28 (2H, m, H-8″), 1.30 (2H, m, H-7″), 1.40 (2H, m, H-7), 1.73 (1H, m, H-2‴), 1.74 (1H, m, H-2′), 1.89 (2H, m, H-6″), 1.96 (2H, m, H-6), 2.41 (1H, m, H-5), 2.45 (1H, m, H-2), 2.68 (1H, dd, *J*=10.3, 10.0 Hz, H-2″), 2.82 (1H, q, *J*=10.3 Hz, H-3″), 2.96 (1H, m, H-1‴), 2.97 (1H, m, H-1‴), 3.15 (1H, m, H-1′), 3.17 (1H, m, H-1′), 3.15 (NH, br t, *J*=6.0 Hz), 5.28 (1H, dd, *J*=15.0, 10.0 Hz, H-4″), 5.53 (NH, br t, *J*=5.7 Hz), 5.56 (1H, ddd, *J*=10.0, 4.3, 2.6 Hz, H-3), 5.63 (1H, m, H-5″), 5.98 (1H, dt, *J*=10.0, 1.85 Hz, H-4); ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 14.04 (C-10″), 14.12 (C-10), 20.10 (C-3′), 20.10 (C-3′), 20.20 (C-3‴), 20.20 (C-3‴), 28.35 (C-2‴), 28.35 (C-7″), 28.56 (C-7), 28.56 (C-2″), 29.02 (C-9″), 29.74 (C-9), 31.38 (C-8″), 31.97 (C-8), 32.32 (C-6″), 32.59 (C-6), 36.92 (C-3″), 37.04 (C-5), 46.74 (C-1‴), 46.96 (C-1′), 49.98 (C-2″), 51.79 (C-2), 124.01 (C-3), 133.05 (C-5″), 131.31 (C-4″), 133.91 (C-4), 173.04 (C-1″), 173.80 (C-1); HRESIMS m/z, 447.3958 $[M+H]^+$, calcd for $C_{28}H_{50}N_2O_2$ 447.3945.

6.10. Cytotoxicity assay

All the isolates and their derivatives were tested for in vitro cytotoxicity on different cancer cell lines by MTT assay. The cell lines used in this study were HELA (cervical cancer), MCF-7 (breast cancer), HEPG2 (liver cancer), and HT-29, COLO-205 (colon cancer). All the cells were obtained from National Center for Cellular Sciences (NCCS), Pune, India. DMEM (Dulbecco's modified Eagles medium), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], trypsin, and EDTA were purchased from sigma chemicals Co., (St. Louis, MO) and Fetal bovine serum was purchased from Gibco. Cells were plated at a density of 10,000 cells per well in 100 μL media in 96 well plates and grown for 24 h. The cells were exposed to a series of concentrations of the test compounds (10–200 μg/mL) for 48 h and the viability of the cells was measured by using MTT method. Briefly, a volume of 10 μL of MTT solution (5 mg/mL in PBS) was added to each well containing 90 μL of the media. The plates were incubated for 4 h at 37 °C. After incubation, a volume of 200 μL of DMSO was added to each well for 10 min at room temperature. Absorbance was measured at 570 nm using multi detection reader (Synergy 4, Biotek, USA). The mean% of cell viability relative to that of untreated cells was estimated from data of three individual experiments. The IC_{50} value of each compound was calculated by curve fitting method.

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