Enzyme Inhibitory Constituents from *Duranta repens*

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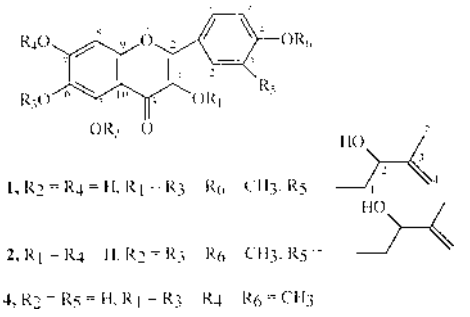
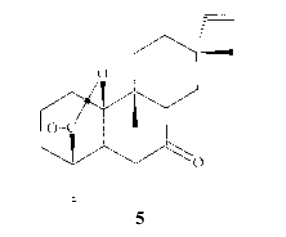
Isoprenylated flavonoids 5,7-dihydroxy-3′-(2-hydroxy-3-methyl-3-butenyl)-3,6,4′-trimethoxyflavone (1), 3,7-dihydroxy-3′-(2-hydroxy-3-methyl-3-butenyl)-5,6,4′-trimethoxyflavone (2) and an isoprenylated acetophenone derivative (3) have been isolated from *Duranta repens* along with known compounds, 5-hydroxy-3,6,7,4′-tetramethoxyflavone (4), rosenonolactone (5), 6,7-dimethoxycoumarin (6), 5α,8α-epidioxyergosta-6,22-dien-3β-ol (7) and 5α,8α-epidioxyergosta-6,9(11),22-trien-3β-ol (8), isolated for the first time from this species. Their structures and the relative configuration were determined by spectroscopic methods (^{1}H- and ^{13}C-NMR, IR, UV and MS) and two-dimensional (2D)-NMR experiments. The compounds 1—5 showed inhibitory activity against prolyl endopeptidase while 4 and 5 were also active against thrombin.

Key words *Duranta repens*; Verbenaceae; isoprenylated flavonoid and acetophenone; enzyme inhibitor

The genus *Duranta* (Verbenaceae) comprises about 35 species which are distributed in tropical and sub-tropical regions. The literature survey revealed that very little phytochemical work has been carried out on the genus *Duranta*, and only some steroids,[1] triterpenes[2] and iridoids[3] have so far been reported. *Duranta repens* Linn. is widely distributed in northern parts of Pakistan and finds various medicinal uses in the indigenous system of medicine. The fruits of this plant afford a medicine for the treatment of malaria.[4] The methanolic extract also shows insecticidal and antifeedant properties.[5] Previously, diterpenoids and flavonoids have been reported.[6] Our preliminary pharmacological screening of the methanolic extract revealed inhibitory activity against both thrombin and prolyl endopeptidase enzymes. This prompted us to carry out the phytochemical studies on this plant. We now report the isolation and structure elucidation of the two new isoprenylated flavonoids **1** and **2**, and of an isoprenylated acetophenone derivative **3**, along with the known compounds, 5-hydroxy-3,6,7,4′-tetramethoxyflavone (**4**),[7] rosenonolactone (**5**),[8] 6,7-dimethoxycoumarin (**6**),[9] 5α,8α-epidioxyergosta-6,22-dien-3β-ol (**7**)[10] and 5α,8α-epidioxyergosta-6,9(11),22-trien-3β-ol (**8**)[11] isolated for the first time from this species.

Prolyl endopeptidase (PEP, EC 3.4.21.26) is the only serine protease which is known to cleave a peptide substrate in the C-terminal side of a proline residue.[12] Prolyl endopeptidase has been suggested to play an important role in the biological regulation of peptide hormones such as vasopresssin, oxytocin, substance P, angiotensin and others.[13] Moreover alterations of PEP enzyme level and activity seems to be associated with several health disorders such as Alzheimer's disease, depression, mania, thrombosis, AIDS and cancer.[14] Specific inhibitors of PEP are expected to have anti-amnesic effects. Many PEP inhibitors have been synthesized as candidates for the treatment of neuropathological disorders.[15] In the course of this work we have evaluated compounds **1**—**5** for their enzyme inhibiting activity. The compounds **1** and **2** showed strong inhibitory activity against PEP while **3**—**5** showed moderate activity. Moreover **4** and **5** also showed strong inhibitory activity against thrombin. The compounds **6**—**8** did not show enzyme inhibiting activity.





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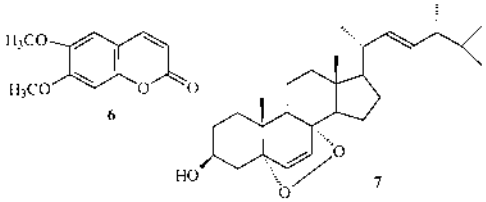

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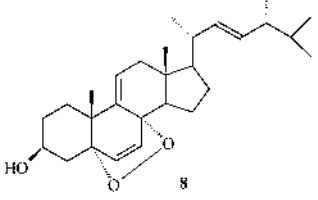

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Results and Discussions

The EtOH extract of shade-dried whole plant of *D. repens* was evaporated *in vacuo*, suspended in H_2O, and successively partitioned with *n*-hexane, $CHCl_3$, and *n*-BuOH. As a result of a series of chromatographic separation, eight compounds have been obtained from the chloroform soluble fraction as described in the Experimental.

Compound **1** was isolated as yellow gummy solid. The molecular formula was assigned as $C_{23}H_{24}O_8$ by high-resolution electron impact (HR-EI)-MS showing $[M]^+$ ion at *m/z* 428.1475. It gave a red color in the Shinoda test,[16] typical for flavonoids, and negative results in the Quastel test indicated the absence of an *ortho* dihydroxyl moiety.[17] The UV spectrum with λ_{max} at 272 and 338 nm, also suggested it to be a flavonoid.[18] The IR spectrum revealed the presence of the hydroxyl groups (3430 cm^{-1}), methoxyl groups (2927, 1193 cm^{-1}) and α,β-unsaturated carbonyl group (1680, 1600 cm^{-1}). The ^{1}H-NMR spectrum provided signals of functional groups including a chelated hydroxyl group (12.94. 1H, s), methoxyl groups [δ 4.01 (3H, s, MeO-6), 3.91 (3H, s, MeO-4′), 3.82 (3H, s, MeO-3)], and an isoprenyl group which was revealed to be 2-hydroxy-3-methyl-3-butenyl by comparison of ^{1}H-NMR spectral data with related compounds[19]; two one-proton double doublet at δ 2.84 (J=13.7, 8.3 Hz) and 3.02 (J=13.7, 4.3 Hz) assignable to 1″ protons, a one-proton double doublet at 4.33 (J=8.3, 4.3 Hz) assignable to a proton at the 2″-position bearing a hydroxyl group, a three-proton singlet at 1.82 (CH_3 at 5″) and two one-proton singlets at 4.83 and 4.90 assignable to an exomethylene (CH_2 at 4″). It also exhibited an ABX system (B ring) [δ 6.98 (1H, d, J=8.7 Hz), 7.90 (1H, d, J=2.3 Hz) and 7.99 (1H, dd, J=8.7, 2.3 Hz)] and one aromatic proton (A ring) [δ 6.54 (1H, s, H-8)]. The broad band and distortionless enhancement by polarization transfer (DEPT) ^{13}C-NMR spectra of **1** corroborated the presence of four methyl, two methylene, five methine and twelve quaternary carbons. The signal at δ 93.2 was typical for 5,6,7-oxygenated C-8 carbon. The EI-MS gave distinct peaks for flavanones at *m/z* 428 ($[M]^+$), 358 ($[M-70]^+$), 357 ($[M-71]^+$), 343 ($[M-70-CH_3]^+$) and two typical daughter fragments at *m/z* 183 ($[A_1+H]^+$) and 219 ($[B_2]^+$). This confirmed the presence of two hydroxyl groups and one methoxyl group in the ring A, and one methoxyl and the side chain in the ring B, the remaining methoxyl group being at C-3. The benzyl cleavage explained the loss of 71 amu while the loss of 70 amu was due to the β-cleavage of the side chain with H-transfer to the aromatic nucleus *via* a 1,6 rearrangement.[20] On the basis of the analysis of its two-dimensional (2D) NMR spectra including heteroatom multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC), **1** was deduced to be a flavanone with an isoprenyl group. The two double doublets of methylene protons of the isoprenylated moiety at δ 2.84 and 3.02 correlated with C-3′ (δ 127.4), C-2″ (δ 75.3), C-2′ (δ 131.3), C-3″ (δ 147.0) and C-4′ (δ 159.6), and a proton geminal to secondary hydroxyl group at δ 4.33 correlated with C-4″ (δ 110.9) and two exo-methylene protons at δ 4.83 and 4.90 coupled with C-2″ (δ 75.3) and C-5″ (δ 18.1) not only confirming the position of double bond but also the attachment of the prenyl moiety to C-3′. The other HMBC correlations are shown in Fig 1. The structure of **1** is, therefore, assigned as 5,7-dihydroxy-3′-(2-hydroxy-3-methyl-3-butenyl)-3,6,4′-trimethoxyflavone).

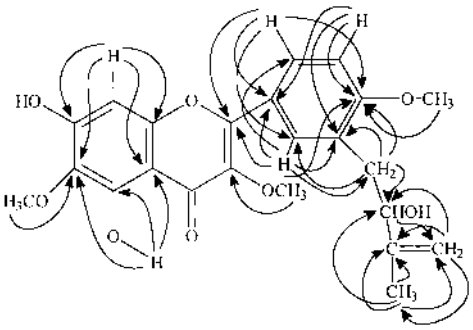

Fig. 1. HMBC Interactions of Compound **1**

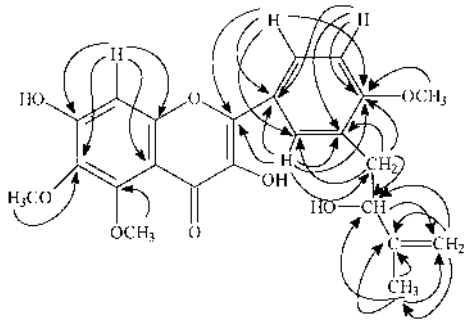

Fig. 2. HMBC Interactions of Compound **2**

Compound **2** was also isolated as yellow gummy solid, assigned the molecular formula $C_{23}H_{24}O_8$ by HR-EI-MS showing molecular ion peak at *m/z* 428.1477. The UV, IR, ^{1}H-NMR and MS showed striking resemblance to those of **1**. The difference between these compounds is interchanged positions of methoxyl and hydroxyl functionalities at C-3 and C-5. It was confirmed by the ^{1}H-NMR spectrum of **2**, in which no signal for 5-OH was observed but a singlet for 5-methoxyl group at δ 4.05 (3H) was present. The other two methoxyl groups at δ 4.02 (3H, s, MeO-6) and δ 3.92 (3H, s, MeO-4′) were identical to compound **1**. The mass spectrum differed from **1** in having fragments at *m/z* 196 $[A]^+$ and 182 ($[A+H-15]^+$) which established that one hydroxyl and two methoxyl groups were present in ring A,[21] the remaining methoxyl and isoprenyl moiety in ring B, and the OH was at C-3. The structure was further confirmed by the HMBC spectrum of **2** shown in Fig 2. The methoxyl group at δ 4.05 was correlated with C-5 (δ 156.2), confirming the location of methoxyl group at C-5 and hydroxyl group at C-3 (δ 138.8). The methylene protons of the isoprenyl chain at δ 2.83 and 3.01 showed correlations with C-4′ (δ 158.6), C-2′ (δ 131.4), C-3′ (δ 127.5), C-3″ (δ 147.2) and C-2″ (δ 75.3) confirming the position of the isoprenyl moiety at C-3′. Consequently, **2** was assigned the structure 3,7-dihydroxy-3′-(2-hydroxy-3-methyl-3-butenyl)-5,6,4′-trimethoxyflavone.

Compound **3** was obtained as yellow oil. The molecular formula was shown to be $C_{15}H_{21}O_4$ by $[M+H]^+$ peak at *m/z* 265.1442 in HR-FAB-MS. The EI-MS spectrum gave a ($[M-CH_2OCH_3]^+$) peak at *m/z* 219 as a base peak followed by $[M-70]^+$ peak at *m/z* 194 due to the loss of the isoprenyl chain from the aromatic ring. The ^{1}H-NMR spectrum provided signals for two methoxyl groups [δ 3.89 (3H, s), δ 3.47 (3H, s)], methylene protons at δ 4.64 (2H, s) and the 2-

Table 1. Enzyme Selectivity [IC_{50} Values (μM)] of Natural Compounds **1**—**5** from *Duranta repens* for Prolyl Endopeptidase and Thrombin

Compound	Prolyl endopeptidase (flavobacterium origin)	Thrombin (bovine source)
	IC_{50}[a] (μM)	IC_{50} (μM)
1	450 ± 0.02	Not active
2	233 ± 0.003	Not active
3	845 ± 0.005	Not active
4	860 ± 0.005	665 ± 0.015
5	675 ± 0.025	875 ± 0.015
Z-Pro-prolinal	0.884 ± 0.025 nM	
Leupeptin		45.4 ± 0.03

a) IC_{50} values are the mean ± standard mean (SEM) error of three assays.

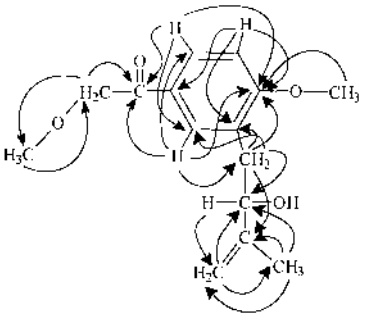

Fig. 3. HMBC Interactions of Compound **3**

hydroxyisoprenyl group[19] [δ 2.79 (1H, dd, J=13.7, 8.6 Hz), 2.97 (1H, dd, J=13.7, 4.1 Hz), 4.30 (1H, dd, J=8.6, 4.1 Hz), 4.82 (1H, s), 4.92 (1H, s), 1.80 (3H, s)]. It also exhibited evidence for an ABX system of the phenyl ring [δ 6.90 (1H, d, J=8.6 Hz), 7.78 (1H, d, J=2.2 Hz) and 7.85 (1H, dd, J=8.6, 2.2 Hz). The broad band and DEPT ^{13}C-NMR spectra of **3** corroborated the presence of three methyl, three methylene, four methine and five quaternary carbons. The signal at δ 194.2 was due to the conjugated carbonyl. On the basis of its 2D NMR spectra (HMQC and HMBC), **3** was deduced to be derived from acetophenone. In the HMBC spectrum, the proton of the phenyl ring at δ 7.78 (H-2′) correlated with C-1 (δ 194.2), C-1″ (δ 36.75), C-6′ (δ 128.7) and C-4′ (δ 162.2), and another phenyl ring proton at δ 7.85 (H-6′) coupled with C-1 (δ 194.2), C-4′ (δ 162.2) and C-2′ (δ 131.2). These results indicated the attachment of carbonyl group directly with phenyl ring. On the other hand, methylenic protons at δ 2.97 and 2.79 (H-1″) correlated with C-3′ (δ 127.4), C-2′ (δ 131.2), C-4′ (δ 162.2) and C-2″ (δ 75.2), confirming the location of the isoprenyl group at C-3′. The methylenic protons at δ 4.64 (H-2) coupled with C-1 (δ 194.2) and methoxyl group at δ 59.3, and the methoxyl group at δ 3.47 also showed interaction with C-2 (δ 75.15) confirming the attachment of the methylene group with both carbonyl and methoxyl groups. Further HMBC interactions shown in Fig. 3 were in accordance to the assigned structure of **3** as 2,4′-dimethoxy-3′-(2-hydroxy-3-methyl-3-butenyl)acetophenone.

Compounds **1**—**5** have been screened against PEP. Their IC_{50} values are shown in Table1 along with the positive control of PEP (Z-Pro-prolinal). The compounds **4** and **5** also showed strong inhibition against thrombin (Table 1) using leupeptin as the positive control. The inhibitors obtained from *Duranta repens* in the present work show IC_{50} values in the range of 233—860 μM, which are strongly active comparing with the previously reported natural inhibitor which shows IC_{50} values in the range of 148—1000 μM.[22] Among these, the new prenylated flavanoids **1** and **2** have shown IC_{50} values of 233 and 450 μM, respectively, while both of them were found to be inactive against thrombin, These compounds were therefore regarded to the selective natural inhibitors towards PEP. On the contrary, compounds **4** and **5** showed low inhibitory activity against PEP with IC_{50} values of 860 and 675 μM, respectively. On the other hand two compounds **4** and **5** also showed inhibitory activity against thrombin with IC_{50} values 665 and 875 μM, respectively.

Experimental

General Optical rotations were taken on a JASCO DIP-360 digital polarimeter. IR spectral data were measured on a JASCO 302-A spectrophotometer with CHCl3. UV spectra were obtained on a Hitachi UV-3200 spectrophotometer. NMR spectra were run on Bruker spectrophotometers, at 400 and 300 MHz instruments. Chemical shifts δ in ppm relative to $SiMe_4$ as internal standard and coupling constant J is described in Hz. EI-, FAB-, and HR-EI-MS were recorded on a JEOL JMS-HX-110 and JMS-DA-500 mass spectrometers, *m/z* (rel. int). Silica gel 60, 70—230 mesh and 200—440 mesh (both from E. Merck) were used for column and flash chromatography, respectively. Silica gel plates (Si 60 F_{254}, E. Merck) were used for TLC.

Chemicals Prolyl endopeptidase (*Flavobacterium meningosepticum* origin) was purchased from Seikagaku Corporation (Tokyo, Japan) and *N*-benzyloxycarbonyl-Gly-Pro-*p*NA was procured from BACHEM Fine Chemicals Co. Specific inhibitor of PEP, *N*-benzyloxycarbonyl-pro-prolinal, was kindly donated by Dr. Hideaki Shimizu, Yakult Central Institute For Microbiological Research, Tokyo, Japan. Bovine thrombin, *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide and leupeptin were purchased from Sigma Chemicals.

Plant Material The whole plant of *Duranta repens* Linn. (Verbenaceae) was collected from District Chitral, N.W.F.P. (Pakistan), in April, 1997, and air-dried. The identity of plant was verified by Prof. M. Qaiser, Department of Botany, University of Karachi. A voucher specimen was deposited in the herbarium of the University of Karachi, Pakistan.

Extraction and Isolation The shade dried plant (20 kg) were extracted with EtOH three times at room temperature and filtered. The filtrate was evaporated *in vacuo* to give a dark greenish residue, which was suspended in water and partitioned successively with *n*-hexane, $CHCl_3$ and *n*-BuOH. The $CHCl_3$ soluble fraction was subjected to medium pressure liquid chromatography (MPLC) using *n*-hexane–$CHCl_3$ in increasing order of polarity to give six fractions. The major fraction obtained from *n*-hexane–$CHCl_3$ (1 : 1) was subjected to column chromatography over silica gel using *n*-hexane–ethylacetate in increasing order of polarity to obtain three major fractions A, B and C. The fraction A obtained from *n*-hexane–ethylacetate (94 : 6) was a binary mixture which was resolved through preparative TLC using *n*-hexane–$CHCl_3$ (3 : 7) as eluent to afforded compounds **4** (25 mg) and **5** (12 mg). The fraction B which eluted with *n*-hexane–ethylacetate (90 : 10) was a mixture of 3 major components. Repeated preparative TLC over silica gel using *n*-hexane–ethylacetate (1 : 1) as eluent provided **6** (6 mg), **7** (8 mg) and **8** (3.6 mg), respectively. The fraction C obtained through elution with *n*-hexane–ethylacetate (88 : 12) was subjected to flash CC over silica gel using *n*-hexane–ethylacetate (70 : 30) as eluent. This operation provided fractions 1—40. The fraction 1—14 showed one major spot which was purified through preparative TLC developing with *n*-hexane–acetone (70 : 30) to obtain compound **3** (6 mg). The fractions 20—28 was also subjected to PTLC in the same solvent system to provide compound **1** (26 mg). Similar work-up with fractions 32—40 yielded compound **2** (8 mg).

5,7-Dihydroxy-3′-(2-hydroxy-3-methyl-3-butenyl)-3,6,4′-trimethoxyflavone) (**1**): Yellow gummy solid, ^{1}H-NMR ($CHCl_3$, 300 MHz) δ: 12.94 (1H, s, OH-5), 7.99 (1H, dd, J=8.7, 2.3 Hz, H-6′), 7.90 (1H, d, J=2.3 Hz, H-2′), 6.98 (1H, d, J=8.7 Hz, H-5′), 6.54 (1H, s, H-8), 4.90 (1H, s, H-4″a), 4.83 (1H, s, H-4″b), 4.33 (1H, dd, J=8.3, 4.3 Hz, H-2″), 4.01 (3H, s, OCH_3-6), 3.91 (3H, s, OCH_3-4′) 3.82 (3H, s, OCH_3-3), 3.02 (1H, dd, J=13.7, 4.3, H-1″a), 2.84 (1H, dd, J=13.7, 8.3, H-1″b), 1.82 (3H, s, CH_3-5″). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 179.13 (s, C-4), 159.6 (s, C-4′), 156.1 (s, C-2), 155.0 (s, C-7), 152.8 (s, C-5), 152.2 (s, C-9), 147.0 (s, C-3″), 138.4 (s, C-3), 131.3 (d, C-2′), 130.0 (s, C-6), 128.7 (d, C-6′), 127.4 (s, C-3′), 122.6 (s, C-1′), 110.9 (t, C-4″), 110.3 (d, C-5′), 106.2 (s, C-10), 93.2 (d, C-8), 75.3 (d, C-2″),

60.9 (q, MeO-6), 60.1 (q, MeO-3), 55.6 (q, MeO-4′), 37.0 (t, C-1″), 18.1 (q, CH_3-5″). IR ν_{max} ($CHCl_3$) cm^{-1}: 3430, 2927, 1680, 1655, 1600, 1193 cm^{-1}. UV λ_{max} (MeOH) nm (log ε): 272 (4.41), 338 (4.48). FAB-MS *m/z*: 429 $[M^+ + H]$. EI-MS *m/z*: 428 $[M]^+$ (78), 413 (72), 358 (100), 357 (87), 343 (59), 342 (5), 219 (5), 183 (10). HR-EI-MS *m/z*: 428.1475 (Calcd for $C_{23}H_{24}O_8$: 428.1471). $[\alpha]_D^{25}$ +18.5° (*c*=0.05, MeOH).

3,7-Dihydroxy-3′-(2-hydroxy-3-methyl-3-butenyl)-5,6,4′-trimethoxyflavone (**2**): Yellow gummy solid, ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 7.96 (1H, dd, *J*=8.6, 2.4 Hz, H-6′), 7.91 (1H, d, *J*=2.4 Hz, H-2′), 6.97 (1H, d, *J*=8.6 Hz, H-5′), 6.56 (1H, s, H-8), 4.92 (1H, s, H-4″a), 4.84 (1H, s, H-4″b), 4.32 (1H, dd, *J*=8.3, 4.2 Hz, H-2″), 4.05 (3H, s, MeO-5), 4.02 (3H, s, MeO-6), 3.92 (3H, s, MeO-4′), 3.01 (1H, dd, *J*=13.8, 4.2 Hz, H-1″a), 2.83 (1H, dd, *J*=13.8, 8.3 Hz, H-1″b), 1.83 (3H, s, CH_3-5″). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 179.6 (s, C-4), 158.6 (s, C-4′), 156.2 (s, C-2), 156.2 (s, C-5), 153.0 (s, C-7), 152.4 (s, C-9), 147.2 (s, C-3″), 138.8 (s, C-3), 131.4 (d, C-2′), 132.1 (s, C-6), 128.8 (d, C-6′), 127.5 (s, C-3′), 122.4 (s, C-1′), 110.8 (t, C-4″), 110.3 (d, C-5′), 106.2 (s, C-10), 94.2 (d, C-8), 75.3 (d, C-2″), 61.8 (q, MeO-5), 61.3 (q, MeO-6), 55.9 (q, MeO-4′), 36.7 (t, C-1″), 18.1 (q, CH_3-5″). IR ν_{max} ($CHCl_3$) cm^{-1}: 3425, 2900, 1684, 1650, 1590, 1190. UV λ_{max} (MeOH) nm (log ε): 272 (4.62), 344 (4.70). FAB-MS: *m/z*: 429 $[M+H]^+$. EI-MS *m/z*: 428 $[M]^+$ (100), 413 (10), 358 (97), 357 (50), 343 (28), 219 (6), 196 (10), 182 (12). HR-EI-MS *m/z*: 428.1477 (Calcd for $C_{23}H_{24}O_8$: 428.1471). $[\alpha]_D^{25}$ +33.3° (*c*=0.03, MeOH).

2,4′-Dimethoxy-3′-(2-hydroxy-3-methyl-3-butenyl)acetophenone (**3**): Yellow oil, ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 7.85 (1H, dd, *J*=8.6, 2.2 Hz, H-6′), 7.78 (1H, d, *J*=2.2 Hz, H-2′), 6.90 (1H, d, *J*=8.6 Hz, H-5′), 4.92 (1H, s, H-4″a), 4.82 (1H, s, H-4″b), 4.64 (2H, s, H-2), 4.30 (1H, dd, *J*=8.6, 4.1 Hz, H-2″), 3.89 (3H, s, MeO-4′), 3.47 (3H, s, MeO-2), 2.97 (1H, dd, *J*=13.7, 4.1 Hz, H-1″a), 2.79 (1H, dd, *J*=13.7, 8.6 Hz, H-1″b), 1.80 (3H, s, CH_3-5″). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 194.2 (s, C-1) 162.2 (s, C-4′), 147.1 (s, C-3″), 131.2 (d, C-2′), 128.7 (d, C-6′), 127.8 (s, C-1′), 127.4 (s, C-3′), 110.2 (t, C-4″), 109.9 (d, C-5′), 75.2 (d, C-2″), 75.15 (t, C-2), 59.3 (q, MeO-2), 55.6 (q, MeO-4′), 36.8 (t, C-1″), 18.0 (q, CH_3-5″). IR ν_{max} ($CHCl_3$) cm^{-1}: 3435, 2927, 1686, 1601, 1259, 1115. UV λ_{max} (MeOH) nm: (log ε) 274 (2.47). EI-MS *m/z*: 219 ($[M-45]^+$) (100), 194 ($[M-70]^+$) (91), 149 (42), 148 (11), 120 (33) 119 (12), 91 (30), 77 (17), 71 (13). HR-FAB-MS (positive) *m/z*: 265.1442 (Calcd for $C_{15}H_{21}O_4$: 265.1439). $[\alpha]_D^{25}$ −25.0° (*c*=0.04, MeOH).

Enzyme Inhibition Assays. PEP Inhibition Assay The PEP inhibition activity was assayed by a modification of the method of Yoshimoto *et al.*[23] 100 mM Tris(hydroxymethyl)aminomethane–HCl buffer containing 1 mM EDTA, pH 7.0, 247 μl, PEP (0.02 unit/300 μl) 15 μl and test sample in 8 μl MeOH, were mixed in 96-well microplate and preincubated for 10 min at 30 °C. The reaction was initiated by adding 30 μl of 2 mM of *N*-benzyloxycarbonyl-Gly-Pro-*p*NA (in 40% 1,4-dioxane) as the substrate. The amount of released *p*-nitroaniline was determined spectrophotometrically, as increase in absorption at 410 nm, with 96-wells microplate reader at 30 °C.

Thrombin Inhibition Assay Enzymatic activity of bovine thrombin (sigma) was measured in a buffer containing 0.145 M NaCl, 0.005 M KCl, 1 mg/ml polyethyleneglycol (PEG-8000), 0.03 M HEPES (pH 7.4), and 0.096 U/well final concentration in the microtiter plate based assay. The enzyme was incubated with the inhibitor at 37 °C for 15 min before starting the reaction. The reaction was initiated with the addition of 0.5 mM *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide. Time dependent optical density changes were followed at 405 nm by a 96 well microplate reader.[6] The IC_{50} values were the average of at least three determinations performed in triplicate.

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