Enzyme Inhibitory Constituents from *Duranta repens*

Itrat ANIS, Saeed AHMED, Abdul MALIK,* Amsha YASIN, and M. Iqbal CHOUDARY

International Center of Chemical Sciences, HEJ Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan. Received November 26, 2001; accepted January 22, 2002

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 (1H- and 13C-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) triterpenes2) and iridoids3) 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 vasopressin, 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.
Results and Discussions

The EtOH extract of shade-dried whole plant of *D. repens* was evaporated *in vacuo*, suspended in H$_2$O, 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, typical for flavonoids, and negative results in the Quastel test indicated the absence of an ortho dihydroxyl moiety. The UV spectrum with $\lambda_{max}$ at 272 and 338 nm, also suggested it to be a flavonoid. The IR spectrum revealed the presence of the hydroxyl groups (3430 cm$^{-1}$), methoxyl groups (2927, 1193 cm$^{-1}$) and $\alpha,\beta$-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 [\(\delta 4.01 (3H, s, MeO-6)\), 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 $\delta$ 2.84 ($J$=13.7, 3.83 Hz) and 3.02 ($J$=13.7, 4.33 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) [\(\delta 6.98 (1H, d, J=8.7Hz)\), 7.90 (1H, d, $J=2.3Hz$) and 7.99 (1H, dd, $J=8.7, 2.3Hz$)] and one aromatic proton (A ring) [\(\delta 6.54 (1H, s, H-8)\)]. The broad band and distortionless enhancement by polarization transfer (DEPT) $^{13}$C-NMR spectra provided signals for flavanones at $\delta$ 127.4, 147.0 and C-2' as a base peak followed by $\delta$ 178.6, 158.6, C-9' ($\delta$ 131.4), C-3' ($\delta$ 132.5), C-2' ($\delta$ 175.3) and C-1' ($\delta$ 174.2) 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.

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 $\delta$ 4.05 (3H) was present. The other two methoxyl groups at $\delta$ 4.02 (3H, s, MeO-6) and 3.92 (3H, s, MeO-3) 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 $\delta$ 4.05 was correlated with C-5 ($\delta$ 156.2), confirming the location of methoxyl group at C-5 and hydroxyl group at C-3 ($\delta$ 138.8). The methoxyl protons of the isoprenyl chain at $\delta$ 2.83 and 3.01 showed correlations with C-4' ($\delta$ 158.6), C-2' ($\delta$ 131.4), C-3' ($\delta$ 127.5), C-2'' ($\delta$ 175.3) confirming the position of the isoprenyl moiety at C-3'. Consequently, 2 was assigned the structure 3,7-dihydroxy-3',6-dimethoxyl-3,6,4'-trimethoxyflavone.

Compound 3 was obtained as yellow oil. The molecular formula was shown to be C$_{19}$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$_3$OCH$_3$]+) 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 [\(\delta 3.89 (3H, s)\), \(\delta 3.47 (3H, s)\)], methylene protons at $\delta$ 3.64 (2H, s) and the 2-
Table 1. Enzyme Selectivity [IC$_{50}$ Values (mM)] of Natural Compounds 1—5 from Duranta repens for Prolyl Endopeptidase and Thrombin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Prolyl endopeptidase (flavobacterium origin)</th>
<th>Thrombin (bovine source)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[IC$_{50}$] (mM)</td>
<td>[IC$_{50}$] (mM)</td>
</tr>
<tr>
<td>1</td>
<td>450 ± 0.02</td>
<td>Not active</td>
</tr>
<tr>
<td>2</td>
<td>233 ± 0.003</td>
<td>Not active</td>
</tr>
<tr>
<td>3</td>
<td>845 ± 0.005</td>
<td>Not active</td>
</tr>
<tr>
<td>4</td>
<td>860 ± 0.005</td>
<td>665 ± 0.015</td>
</tr>
<tr>
<td>5</td>
<td>675 ± 0.025</td>
<td>875 ± 0.015</td>
</tr>
<tr>
<td>Z-Pro-prolinal Leupeptin</td>
<td>0.884 ± 0.025 nm</td>
<td>45.4 ± 0.03</td>
</tr>
</tbody>
</table>

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

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

**Duranta repens** in the present work show IC$_{50}$ values in the range of 233—860 μM, which are strongly active compared with the previously reported natural inhibitor which shows IC$_{50}$ values in the range of 148—1000 μM. 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 CHCl$_3$. 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$_3$ as internal standard and coupling constant J is described in Hz. EI-, FAB-, and HR-El-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—400 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) was purchased from Seikagaku Corporation (Tokyo, Japan) and N-benzyloxy carbonyl-Gly-Pro-p-nitroaniline and leupeptin were purchased from Sigma Chemicals.

**Material** The whole plant of Duranta repens L. (Verbenaceae) was collected from District Chitrail, 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$_3$ solubel 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–ethanol in increasing order of polarity to obtain three major fractions A, B and C. The fraction A obtained from n-hexane–ethanolacetate (94:6) was a binary mixture which was resolved through preparative TLC using n-hexane–CHCl$_3$ (3:7) as eluent to afford compounds 4 (25 mg) and 5 (12 mg). The fraction B which eluted with n-hexane–ethanolacetate (90:10) was a mixture of 3 major components. Repeated preparative TLC over silica gel using n-hexane–ethanolacetate (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–ethanolacetate (88:12) was subjected to flash CC over silica gel using n-hexane–ethanolacetate (70:30) as eluent. This operation provided fractions 1—14. The major compound 14 was then purified through preparative TLC developing with n-hexane-aceton (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 (I): Yellow gummy solid, δ-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, d, J = 8.7 Hz, H-4'), 4.90 (1H, s, H-4), 4.83 (1H, s, H-4b), 4.33 (1H, dd, J = 8.3, 4.3 Hz, H-2'), 4.01 (3H, s, OCH$_3$-6), 3.91 (1H, s, OCH$_2$-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'), 13C-NMR (CDCl$_3$, 125 MHz) δ: 179.13 (s, C-4), 159.6 (s, C-40), 131.2 (s, C-4), 128.7 (s, C-5), 110.3 (s, C-6), 106.2 (s, C-7), 95.3 (s, C-8), 75.3 (s, C-2), 127.4 (s, C-3), 134.8 (s, C-3), 131.3 (s, 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), 95.3 (d, C-8), 75.3 (d, C-2'), 45.4 (s, C-13), 42.0 (s, C-15), 36.75 (s, 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, CH2-7'), IR νmax (CHCl3) cm⁻¹: 3430, 2927, 2919, 1686, 1601, 1259, 1115 (m). UV λmax (MeOH) nm (log e): 272 (4.41), 338 (4.48). FAB-MS m/z: 429 [M⁺+H]⁺. EI-MS m/z: 428 [M⁺]² (78), 413 (72), 357 (80), 37 (84), 342 (5), 219 (5), 183 (10). HR-EL-MS m/z: 428.1475 (Calcd for C23H24O8: 428.1474). [α]D² = +18.5° (c = 0.05, MeOH).

3,5-Dihydroxy-2'-5'-di(hydroxy-3-methyl-3-butenyl)-6,4'-trimethoxy-flavone (2): Yellow gummy solid. ¹H-NMR (CDCl3, 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'), 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-4), 3.92 (3H, s, MeO-3), 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, CH3-5'). ¹³C-NMR (CDCl3, 100 MHz) δ: 179.6 (s, C-7), 158.6 (s, C-4), 156.2 (s, C-2), 156.2 (s, C-5), 153.0 (s, C-3), 152.4 (s, C-9), 147.2 (s, C-3'), 138.8 (s, C-3), 131.4 (d, C-2'), 131.2 (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, CH3-5'). IR νmax (CHCl3) cm⁻¹: 3425, 2900, 1684, 1650, 1590, 1190. UV λmax (MeOH) nm (log e): 272 (4.62), 344 (4.70). FAB-MS: m/z: 429 [M⁺+H]⁺.

Enzyme Inhibition Assays. PEP Inhibition Assay: The PEP inhibition activity was assayed by a modification of the method of Yoshimoto et al. ¹) 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-benzylxoyacarbonyl-Gly-Pro-pNA (in 40% 1,4-dioxane) as the substrate. The amount of released p-nitroaniline was determined spectrophotometrically, as increase in absorbance 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 polyethylene glycol (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-Phenyl-Val-Arg-p-nitroanilide. Time dependent optical density changes were followed at 405 nm by a 96 well microplate reader. ²) The IC₅₀ values were the average of at least three determinations performed in triplicate.

References and Notes