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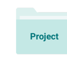

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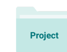

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Biosci. Biotechnol. Biochem., **69** (9), 1763–1766, 2005

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Note

Prolyl Endopeptidase and Thrombin Inhibitory Diterpenoids from the Bark of *Xylopia aethiopica*

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Received November 30, 2004; Accepted May 15, 2005

The inhibitory effects of seven diterpenes, belonging to three different structural classes and isolated from the bark of *Xylopia aethiopica*, were investigated against the enzymes prolyl endopeptidase (PEP) and α-thrombin. Five compounds exhibited inhibitory activity against them.

Key words: *Xylopia aethiopica*; diterpene; prolyl endopeptidase; thrombin; enzyme inhibition

Enzyme inhibition is an important area of pharmaceutical research which has already led to the discovery of a wide variety of drugs that are useful for treating a number of diseases.[1)] Prolyl endopeptidase (PEP, EC 3.4.21.26) is a member of a new class of serine peptidases (serine protease) which catalyses the hydrolysis of peptide bonds at the *L*-proline carboxy terminal and thus plays an important role in the biological regulation of proline-containing neuropeptides and peptide hormones which have been recognized to be involved in learning and memory.[2,3)] Efforts have recently been made to search for new PEP inhibitors as anti-dementia drugs.[3,4)]

The enzyme, α-thrombin (EC 3.4.21.5), also belongs to the serine protease family, and regulates platelet aggregation, endothelial cell activation and other important responses in vascular biology.[5)] The inhibition of thrombin is an important tool against thrombosis and arteriosclerosis, as well as in the prevention of myocardial infarction.[6)]

In our search of PEP and thrombin inhibitors, we have recently evaluated diterpenes isolated from the bark of *Xylopia aethiopica* (Annonaceae) which is a medicinal plant widely distributed in Western Africa. A decoction of its bark is used in the treatment of dysentery, bronchitis and biles born disease. This disease is a rare condition characterized by a progressive inflammatory process that begins very soon after birth. The bile ducts outside the liver are usually affected first, attacked by inflammatory cells, resulting in damage and eventual disappearance. During this process, bile is trapped inside the liver and rapidly causes damage and scarring to the liver cells and then cirrhosis.[7,8)]

Our work on the combined hexane and ethyl acetate extract of the bark of *Xylopia aethiopica* has resulted in the isolation of five known compounds: kolavenic acid (**1**), 2-oxo-kolavenic acid (**2**), *ent*-kaur-16-en-19-oic acid (**3**), trachyloban-19-oic acid (**4**) and 7β-hydroxytrachyloban-19-oic acid (**5**). Methyl trachyloban-19-oate (**6**)[9)] and benzyl trachyloban-19-oate (**7**) were obtained by the esterification of trachyloban-19-oic acid (**4**) with diazomethane and benzyl bromide, respectively (Fig. 1).[10–12)]

Xylopia aethiopica A. Rich. was collected in February 1994 from the Douala-Edea forest reserve (Cameroon). The plant was identified, and a voucher specimen (# 55011) was deposited, by Dr. Achoundong of National Herbarium, Yaounde (Cameroon). Dried and finely powdered plant material (10 kg) was successively extracted with hexane (20-liter × 3 times), EtOAc (20-liter × 3 times) and MeOH (20-liter × 3 times) at room temperature. After filtration and removal of each solvent, the hexane and EtOAc extracts were combined. This combined extract (100 g) was chromatographed in a 70–230 mesh silica gel column (2.5 kg) with stepwise gradient elution by hexane/EtOAc (100:0; 95:5; 85:15; 70:30; 50:50; 25:75; 0:100). Individual column fractions 1–140, each containing 250 ml, were collected and combined according to their TLC profiles on precoated Kieselgel 60 F_{254} plates developed with a hexane/EtOAc mixture. Seven important groups of fractions A (1–24), B (25–60), C (61–79), D (80–93), E (94–100), F (101–114) and G (115–140), respectively, were eluted. Fraction A (30 g) upon recrystallisation from hexane,

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Abbreviations: PEP, prolyl endopeptidase; *p*NA, paranitroanilide

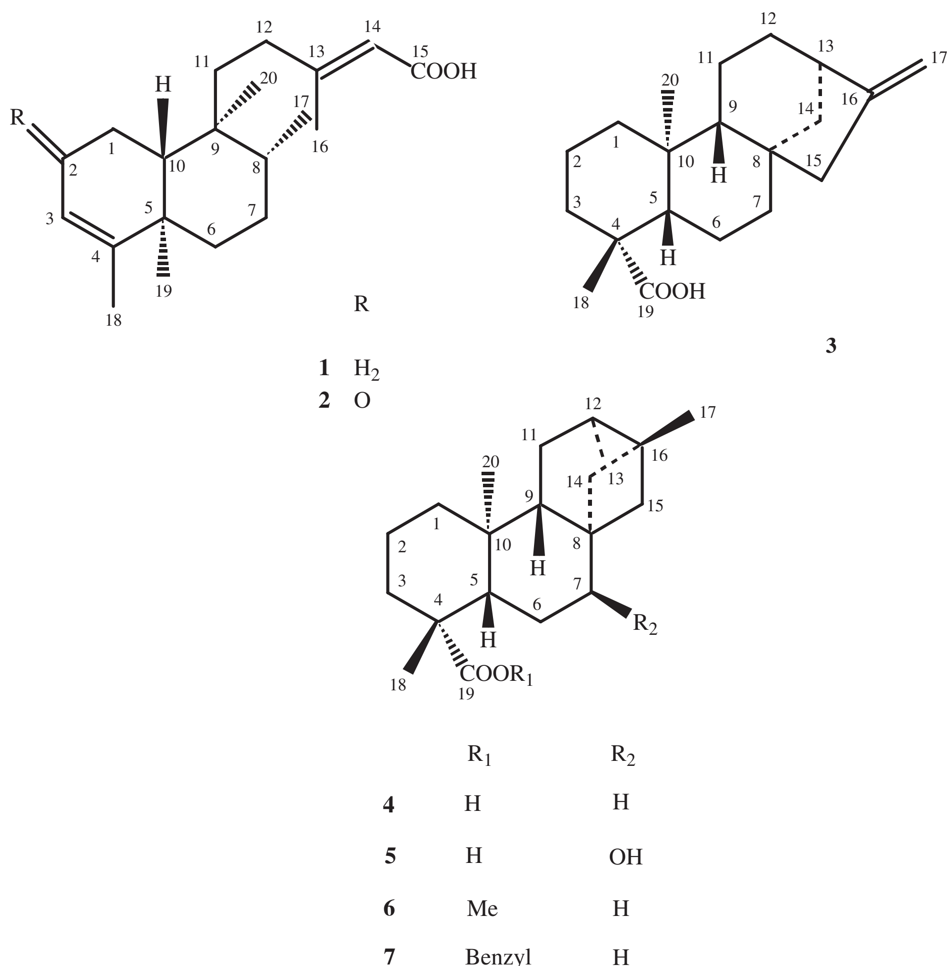

Fig. 1. Structures of the Diterpenes (**1–5**) from *X. aethiopica* and Their Derivatives (**6–7**).

yielded a mixture of compounds **3** and **4**, while fractions B (10 g) and D (4 g) afforded compounds **1** and **2** upon respective recrystallisation from 80:20 and 70:30 hexane/EtOAc. Fraction G (500 g) was rechromatographed on a silica gel column, using CH_2Cl_2/MeOH gradient elution, to obtain compound **5** as colorless needles in MeOH; mp 128–129° C. Known phytosterol and β-sitosterol-3-*O*-β-D-glucopyranoside were also isolated from fraction G. Compounds **1–5** were identified by comparing their physical and spectroscopic data with those reported in the literature[9–12] and by TLC comparison with authentic samples. Compound **6** was synthesized by dissolving trachyloban-19-oic acid (**4**; 2 g, 6.62 mmol) in anhydrous ether (10 ml) and adding an Et_2O solution of diazomethane (7 mmol) at room temperature. After working up, the product was rechromatographed in a silica gel column by using hexane and ethyl acetate to obtain compound **6** (1.98 g) in a 95% yield as a colorless powder, R_f 0.30 (hexane/EtOAc, 98:2). A mixture of trachyloban-19-oic acid (**4**; 2 g, 6.62 mmol) and K_2CO_3 (1 g, 7.2 mmol) in anhydrous DMSO (40 ml) was stirred at room temperature for 30 min. Benzyl bromide (0.84 ml, 7.2 mmol) was then added, and the mixture stirred at room temperature for 12 h. The mixture was partitioned between Et_2O and aq 0.5 N HCl (20 ml). The organic phase was washed with H_2O (20 ml × 3 times) and dried over $MgSO_4$ (10 g). The solvent was removed by evaporation at reduced pressure, and a colorless liquid residue was obtained and rechromatographed in the silica gel column. Elution with hexane/EtOAc (98:2) yielded compound **7** in a 94% yield as a colorless powder, R_f 0.30 (hexane/EtOAc, 98:2).

Compound **6**: $C_{21}H_{32}O_2$; R_f 0.30 (hexane/EtOAc = 98:2); mp 92.5 °C; ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.54 (1H, m, H-12), 0.73 (3H, m, CH_3-20), 0.80 (1H, m, H-13), 1.12 (3H, s, CH_3-17), 1.17 (3H, s, CH_3-18), 3.60 (3H, s, COOMe); ^{13}C-NMR (90 MHz, $CDCl_3$) δ: 39.4 (C-1), 18.7 (C-2), 37.8 (C-3), 43.6 (C-4), 56.9 (C-5), 21.7 (C-6), 39.2 (C-7), 40.7 (C-8), 52.7 (C-9), 38.9 (C-10), 19.7 (C-11), 20.5 (C-12), 24.2 (C-13), 33.1 (C-14), 50.1 (C-15), 22.4 (C-16), 20.5 (C-17), 28.9 (C-18), 184.7 (C-19), 12.4 (C-20), 51.2 (OCH_3).

Compound **7**: $C_{27}H_{36}O_2$; R_f 0.30 (hexane/EtOAc = 98:2); mp 76.2 °C; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.52 (1H, m, H-12), 0.74 (3H, m, CH_3-20), 0.79 (1H, m, H-13), 1.10 (3H, s, CH_3-17), 1.15 (3H, s, CH_3-18), 5.07 and 5.14 (1H each, ABd, J = 12.4 Hz, benzyl protons),

Table 1. *In Vitro* Quantitative Inhibition of PEP and Thrombin by Compounds **1**–**7**

Compound	Prolyl endopeptidase			Thrombin		
	Concentration (μM)	% inhibition[a]	IC_{50}[b] (μM)	Concentration (μM)	% inhibition[a]	IC_{50}[b] (μM)
1	—	—	99.01 ± 1.83	—	—	64.78 ± 2.98
2	1000	34.39	—	1000	NA	—
3	—	—	60.23 ± 2.87	250[c]	17.58	—
4	—	—	164.00 ± 1.82	250[c]	44.48	—
5	1000	NA	—	1000	NA	—
6	—	—	45.00 ± 0.40	—	—	39.82 ± 3.19
7	—	—	36.74 ± 1.61	—	—	37.46 ± 0.58
Bacitracin[d]	—	—	129.26 ± 3.30	—	—	—
Leupeptin[e]	—	—	—	—	—	45.40 ± 3.01

All reactions were performed in 96-well microplates of a SpectraMax 340 instrument (Molecular Devices, U.S.A.).
[a]Values were calculated by the formula $[(E - S)/E] \times 100$, where E is the activity of the enzyme without a test compound and S is the activity of the enzyme with the test compound.
[b]Each IC_{50} value is the mean $\pm$ standard error of the mean (SEM) of three assays and was calculated by the EZ-Fit enzyme kinetics program (Perrella Scientific, Inc., Amherst, U.S.A.).
[c]These concentrations showed poor solubility in the thrombin buffer when added > 250 μM.
[d]Positive control for PEP.
[e]Positive control for thrombin.
NA, not active.
"—" These compounds showed more than 50% inhibition of both the enzymes (PEP and thrombin) at 1.0 mM. They were subsequently screened for their IC_{50} values, and the results are presented in the table. The IC_{50} values were not measured for those compounds which showed less than 50% inhibitory activity at 1.0 mM and only the percentage inhibition of the enzymes at 1.0 mM concentration are given in the table.

7.27–7.40 (5H, m).

The inhibition activity of prolyl endopeptidase (PEP, from *Flavobacterium meningosepticum*, 0.02 unit/well; Seikagaku Corporation Tokyo, Japan) was assayed in 247 μl of a *Tris* HCl buffer (100 mm, pH 7.0, containing 1 mm EDTA) and 15 μl of PEP (0.02 unit/300 μl). The test sample of 8 μl in MeOH was preincubated with the enzyme at 30° C for 10 min. The reaction was then initiated by adding 30 μl of 0.2 mM of *N*-benzyloxy-carbonyl-Gly-Pro-*p*NA (in 40% 1,4-dioxane) as the substrate. The amount of released *p*-nitroaniline was measured spectrophotometrically at 410 nm by a 96-well microplate spectrophotometer (Spectramax 340, Molecular Devices, U.S.A.).

The enzymatic activity of thrombin (from bovine origin, Sigma) was measured in 150 μl of a buffer containing 0.145 M NaCl, 0.005 M KCl, 1 mg/ml of polyethylene glycol (PEG-8000), 0.03 M HEPES (pH 7.4) and 20 μl thrombin (the final concentration of the enzyme in the assay was 0.096 units). The inhibitor (10 μl) was preincubated with the enzyme mixture at 37° C for 15 min before initiating the reaction by the addition of 20 μl of 0.5 mM *N*-benzoyl-Phe-Val-Arg-*p*NA. The time-dependent changes in the optical density were measured at 405 nm with a microplate reader (Spectramax 340).[13,14]

The percentage inhibition and/or IC_{50} values are shown in Table 1, together with the values for the positive controls of PEP (bacitracin) and thrombin (leupeptin). *In vitro* enzyme assays of the seven compounds showed that compounds **1**, **3**, **4**, **6** and **7** possessed dose-dependent inhibitory activity against PEP as compared to positive control bacitracin ($IC_{50} = 129.26 \pm 3.30$ μM), while compound **2** showed less inhibitory activity against PEP. Compounds **1**, **6** and **7** also showed significant inhibitory activity against thrombin when compared with positive control leupeptin ($IC_{50} = 45.40 \pm 3.01$ μM).

Compound **1** showed significant activity compared to compound **2** that contains a carbonyl group at C-2 position. Compound **5** was found to be inactive due to the presence of a hydroxyl group at C-7 in comparison to compound **4**. The enzyme-inhibition potential was enhanced when methyl and benzyl esters of compound **4** were synthesized.

Acknowledgments

We are grateful to Mr. Koji Yoshinaga and Mr. Naoyoshi Miura of the Central Research Laboratories of Zeria Pharmaceutical Co., Ltd. for their help in the establishment of the PEP assay.

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