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Novel direct factor Xa inhibitory compounds from *Tenebrio molitor* with anti-platelet aggregation activity

Wonhwa Lee [a, 1], Mi-Ae Kim [a, b, 1], InWha Park [c, 1], Jae Sam Hwang [b], MinKyun Na [c, *], Jong-Sup Bae [a, **]

[a] College of Pharmacy, CMRI, Research Institute of Pharmaceutical Sciences, BK21 Plus KNU Multi-Omics based Creative Drug Research Team, Kyungpook National University, Daegu 41566, Republic of Korea
[b] Department of Agricultural Biology, The National Academy of Agricultural Science, RDA, Wanju-gun 55365, Republic of Korea
[c] College of Pharmacy, Chungnam National University, Daejeon 34134, Republic of Korea

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ABSTRACT

Tenebrio molitor is an edible insect that has antimicrobial, anticancer, and antihypertensive effects. The aim of this study was to identify the unreported bioactive compounds from *T. molitor* larvae with inhibitory activities against factor Xa (FXa) and platelet aggregation. Isolated compounds were evaluated for their *anti*-FXa and anti-platelet aggregation properties by monitoring clotting time, platelet aggregation, FXa activity, and thrombus formation. A diketopiperazine (**1**, cyclo($_L$-Pro-$_L$-Tyr)) and a phenylethanoid (**2**, *N*-acetyltyramine) were isolated and inhibited the catalytic activity of FXa in a mixed inhibition model and inhibited platelet aggregation induced by adenosine diphosphate (ADP) and U46619. They inhibited ADP- and U46619-induced phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) and the expression of P-selectin and PAC-1 in platelets. They also improved the production of nitric oxide and inhibited the oversecretion of endothelin-1 compared to that of the ADP- or U46619-treated group. In an animal model of arterial and pulmonary thrombosis, the isolated compounds showed enhanced antithrombotic effects. They also elicited anticoagulant effects in mice. Compounds **1**–**2** inhibited ADP-, collagen-, or U46619-induced platelet aggregation and showed similar anti-thrombotic efficacy to rivaroxaban, a positive control. Therefore, 1–2 could serve as candidates and provide scaffolds for the development of new *anti*-FXa and anti-platelet drugs.

1. Introduction

Thrombosis is the leading cause of death worldwide and plays a pivotal role in the pathogenesis of numerous cardiovascular diseases, including acute coronary syndrome and deep vein thrombosis (Fares, 2013). Most thromboembolic processes require anticoagulant therapy, which explains the current efforts to develop specific and potent antithrombotic agents (Fares, 2013). Developing direct thrombin inhibitors was the focus of early antithrombotic drug development efforts and is indicative of the central role thrombin plays in thrombosis (Ostrem et al., 1998). However, a clinical study showed that the continuous production of thrombin from prothrombin was not blocked by direct inhibitors of thrombin (Philippides and Loscalzo, 1996). It is necessary to inhibit high concentrations of thrombin *in vivo* to produce an efficient antithrombotic activity, which can lead to undesirable anticoagulation and an accompanying risk of increased complications from hemorrhage. Therefore, the selective inhibition of coagulation factors located upstream of thrombin may be safer owing to the reduction in bleeding risk. In this context, factor Xa (FXa) has recently emerged as a more attractive target for the development of new anticoagulants (Bauer, 2006). In addition, inhibition of FXa may prevent the continuous production of thrombin, while maintaining its basal activity for primary hemostasis (Bauer, 2006).

Platelet activation in atherosclerotic arteries leads to arterial thrombosis; hence, a precise regulation of platelet function is imperative to prevent thrombotic events (Ruggeri, 2002). The insufficient antithrombotic and antiplatelet activities of currently available drugs may cause relapse in patients with cardiovascular

* Corresponding author. College of Pharmacy, Chungnam National University, 99 Daehak-ro, Yuseong, Daejeon 34134, Republic of Korea.
** Corresponding author. College of Pharmacy, Kyungpook National University, 80 Daehak-ro, Buk-gu, Daegu 41566, Republic of Korea.
E-mail addresses: mkna@cnu.ac.kr (M. Na), baejs@knu.ac.kr (J.-S. Bae).
[1] These authors contributed equally to this work.

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diseases. This explains the efforts to develop protective and therapeutic anticoagulant and antithrombotic agents. Research is highly needed on novel bioactive compounds and drugs with different mechanisms of action, increased efficacy, and low toxicity (Fares, 2013). Upon exposure to activating agonists (e.g., thrombin, adenosine diphosphate (ADP), and collagen), platelets liberate arachidonic acid stored as phospholipid in the platelet plasma membrane, which is converted into thromboxane A_2 by the sequential activities of cyclooxygenase and thromboxane A_2 synthase (Malmsten, 1986; Samuelsson et al., 1978). The released thromboxane A_2 acts as a positive feedback mediator in the activation and recruitment of more platelets to the primary hemostatic plug (Hourani and Cusack, 1991), which results in the formation of a platelet plug. Thromboxane A_2 exerts its actions via specific G protein-coupled receptors (GPCRs) and has been described as a potent platelet agonist (Hourani and Cusack, 1991; Shen and Tai, 1998).

Research into novel foods and medicinal materials has focused on insect resources, demonstrating their potential value. For example, *Lumbricus rubellus* earthworm powder has antithrombotic and anticoagulant activities (Hahn et al., 1997). Further, small molecule alkaloids isolated from *Scolopendra subspinipes mutilans* and *Protaetia brevitarsis seulensis* inhibit platelet aggregation and thrombus formation (Lee et al., 2016). *Tenebrio molitor* Linnaeus is a popular food in China, Cambodia, Thailand, and Mexico and is distributed worldwide (Youn et al., 2014). The Food and Agriculture Organization (FAO) of the United Nations has reported that beetles, including *T. molitor* (Family: Tenebrionidae, Order: Coleoptera), are the most consumed edible insects worldwide (Van et al., 2013). In addition, the Ministry of Food and Drug Safety (MFDS) of Korea approved *T. molitor* larvae as a food resource. The biological activities of *T. molitor* have been reported to include antimicrobial, anticancer, antidementia, and antihypertensive effects (Dai et al., 2013; Moon et al., 1994; Youn et al., 2014). However, the *anti*-FXa and anti-platelet effects of small-molecule isolates from *T. molitor* larvae have not yet been studied. In this study, the chemical investigation of mealworms resulted in the isolation of a diketopiperazine (**1**, cyclo($_L$-Pro-$_L$-Tyr)) and a phenylethanoid (**2**, *N*-acetyltyramine). The goal of the present study was to analyze the effects of the small molecule compounds isolated from *T. molitor* larvae on blood clotting time, FXa activity, and platelet functions. Their antithrombotic activities were further characterized in animal models. To the best of our knowledge, this is the first report of the *anti*-FXa and anti-platelet effects of *T. molitor* larvae and the discovery of new compounds proving dual inhibition of FXa and platelet aggregation.

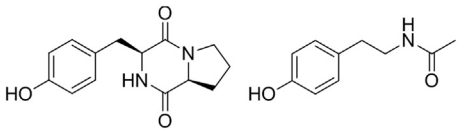
2. Materials and methods

The online supplement provides information on the methods used in this study.

3. Results

3.1. Isolation and structural determination of small-molecule isolates from T. molitor

Freeze-dried larvae of *T. molitor* were refluxed with 1% acetic acid in 50% EtOH. The concentrated extract was suspended in water and partitioned sequentially with *n*-hexane, CH_2Cl_2, and EtOAc. The EtOAc fraction was isolated using various chromatographic techniques, leading to the purification of a diketopiperazine (**1**, cyclo(L-Pro-L-Tyr)) and a phenylethanoid (**2**, *N*-acetyltyramine). The structure of each compound (Fig. 1) was determined using spectroscopic techniques, especially the detailed analyses of their NMR spectra. Based on NMR and HPLC (using an evaporative light scattering detector (ELSD)) analyses, the purity of compounds **1** and **2** was determined to be 95% and 97%, respectively (see Fig. S8-S10).



1, cyclo(L-Pro-L-Tyr) 2, *N*-acetyltyramine

Fig. 1. Structure of isolated compounds from *Tenebrio molitor*.

Compound **1**, cyclo($_L$-Pro-$_L$-Tyr), was isolated as a colorless amorphous solid, $[\alpha]_D^{20}$ -56.4 (*c* 0.33, MeOH). The presence of a diketopiperazine system in cyclo($_L$-Pro-$_L$-Tyr) was deduced from the characteristic ^{1}H NMR chemical shifts for two α-protons (δ 4.05, 4.36) and ^{13}C NMR chemical shifts for two amide carbonyl groups (δ 167.0, 170.8). Two aromatic proton signals at δ 7.04 (2H, d, J = 8.5 Hz, H-2′, and H-6′) and 6.70 (2H, d, J = 8.5 Hz, H-3′ and H-5′), as well as a methylene proton signal at δ 3.06, suggested that one amino acid was a tyrosine. The methylene proton resonances at δ 3.55–1.23 implied that cyclo($_L$-Pro-$_L$-Tyr) has a proline residue. The absolute configuration of compound **1** was determined from the negative specific rotation and was identified as a cyclo($_L$-Pro-$_L$-Tyr) (Mehnaz et al., 2013).

Compound **2**, *N*-acetyltyramine, was isolated as a colorless amorphous solid. ^{1}H NMR spectrum showed signals indicating two aromatic protons with an *ortho* coupling system [δ 7.01 (2H, d, J = 8.5 Hz, H-2, H-6), 6.69 (2H, d, J = 8.5, H-3, H-5)], two methylene protons [δ 3.31 (2H, m, H_2-8), 2.67 (2H, t, J = 7.4 Hz, H_2-7)], and an acetyl proton [δ 1.90 (3H, s, H_3-11)]. The ^{13}C NMR spectrum displayed a carbonyl group [δ 173.2 (C-10)] and one benzene ring bearing one oxygenated carbon [δ 157.1 (C-1)]. Thus, compound **2** was identified as *N*-acetyltyramine, which was confirmed by comparing the spectroscopic data with those previously reported in the literature (Zhang et al., 2011).

3.2. Effect of isolated compounds on clotting time in vitro and ex vivo

To evaluate the effects of cyclo($_L$-Pro-$_L$-Tyr) (**1**) and *N*-acetyltyramine (**2**) on coagulation parameters *in vitro*, we measured their effects on activated partial thromboplastin time (aPTT) and prothrombin time (PT). Rivaroxaban, a commercially available FXa inhibitor, was used as the positive control. As shown in Table 1, pre-administration of each compound *i.v.* to mice significantly increased aPTT in a dose-dependent manner at doses ranging from 26 to 260 μg/kg (cyclo($_L$-Pro-$_L$-Tyr)) and 90–179 μg/kg (*N*-acetyltyramine). At 2.92, 2.72, and 5.21 μM, rivaroxaban, cyclo($_L$-Pro-$_L$-Tyr), and *N*-acetyltyramine, respectively, doubled the clotting time of *in vitro* aPTT assays. The average circulating blood volume of mice is 72 mL/kg (Diehl et al., 2001). Because the average weight of the mice used in this study was 27 g, and the average blood volume was 2 mL, the concentration of cyclo($_L$-Pro-$_L$-Tyr) (26, 130, or 260 μg/kg) and *N*-acetyltyramine (18, 90, or 179 μg/kg) equaled a peripheral blood concentration of approximately 1, 5, and 10 μM, respectively. However, the PT was not significantly higher in mice treated with each compound than it was in mice treated with vehicle only (data not shown).

To confirm the above *in vitro* results in an *ex vivo* assay, each group (n = 5) received *i.v.* injections of the indicated compound for 4 consecutive days. Each compound significantly prolonged the blood clotting time in a dose-dependent manner as shown in Table 1. At 2.46, 2.43, and 3.40 μM, rivaroxaban, cyclo($_L$-Pro-$_L$-Tyr),