ภาคผนวก 4 : ผลงานวิจัยตีพิมพ์ เรื่อง Anti-HIV-1 protease activity of compounds from *Boesenbergia pandurata.*

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Bioorganic & Medicinal Chemistry 14 (2006) 1710–1714

Anti-HIV-1 protease activity of compounds from *Boesenbergia pandurata*

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Received 15 September 2005; revised 10 October 2005; accepted 11 October 2005
Available online 2 November 2005

Abstract—Searching for anti-HIV-1 protease (PR) inhibitors of Thai medicinal plants led to the isolation of a new cyclohexenyl chalcone named panduratin C (**1**) and chalcone derivatives (**2**–**6**) from the methanol extract of *Boesenbergia pandurata* rhizomes. The known compounds were identified to be panduratin A (**2**), hydroxypanduratin A (**3**), helichrysetin (**4**), 2′,4′,6′-trihydroxyhydrochalcone (**5**), and uvangoletin (**6**). The structures of all compounds were elucidated on the basis of chemical and spectroscopic methods. It was found that **3** possessed the most potent anti-HIV-1 PR activity with an IC_{50} value of 5.6 μM, followed by **2** (IC_{50} = 18.7 μM), whereas other compounds exhibited only mild activity. Structure–activity relationships of these compounds on anti-HIV-1 PR activity are summarized as follows: (1) hydroxyl moiety at position 4 conferred higher activity than methoxyl group; (2) prenylation of dihydrochalcone was essential for activity; (3) hydroxylation at position 4‴ reduced activity; and (4) introduction of double bond at C1′ and C6′ of chalcone gave higher activity. As regards active constituents contained in *B. pandurata* rhizomes, hydroxypanduratin A (**3**) and panduratin A (**2**) are active principles against HIV-1 PR.

1. Introduction

Boesenbergia pandurata Holtt., locally known in Thai as Kra-chai, is a perennial herb belonging to the Zingiberaceae family. The fresh rhizomes have a characteristic aroma and a slightly pungent taste. It is commonly used in Southeast Asia as a food ingredient, a folk medicine for the treatment of several diseases such as aphthous ulcer, dry mouth, stomach discomfort, leukorrhea, and dysentery.[1] The rhizomes contain essential oil,[2] pinostrobin, cardamonin, boesenbergin,[3] 5,7-dimethoxyflavone, 1,8-cineole, and panduratin.[4] In the primary health care project of Thailand, the rhizomes of this plant are used for treatment of dyspepsia. Moreover, it has also been used as self-medication by AIDS patients in Thailand. As regards its biological activities, *B. pandurata* exhibits antibacterial,[5] antifungal,[6] anti-inflammatory, analgesic, antipyretic,[7] antispasmodic,[8,9] antitumor,[10] and insecticidal activities.[11]

The human immunodeficiency virus type-1 (HIV-1), a member of retrovirus family, has been a causative organism in an acquired immunodeficiency syndrome (AIDS). One of the important enzymes necessary for the replication of this virus is HIV-1 protease (HIV-1 PR). HIV-1 PR belongs to the aspartyl protease class and functions as a dimer of 99 amino acids each. This enzyme plays a crucial role in the process of viral maturation and infectivity.[12] Thus, searching for HIV-1 PR inhibitors from natural sources has become a promising approach.

In the previous study, we reported the activity of some compounds isolated from *B. pandurata* on anti-HIV-1 PR activity.[13] Herein, we report the isolation, structure elucidation of a new compound, and the activity against HIV-1 PR of chalcone derivatives from this plant.

2. Results and discussion

The MeOH extract of rhizomes of *B. pandurata* was fractionated by silica gel column chromatography and preparative TLC to obtain one new cyclohexenyl

Keywords: HIV-1 protease; Cyclohexenyl chalcone derivatives; *Boesenbergia pandurata*.
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doi:10.1016/j.bmc.2005.10.019

chalcone (panduratin C, **1**) together with five known chalcones, panduratin A (**2**),[14] hydroxypanduratin A (**3**),[15] helichrysetin (**4**),[16] 2′,4′,6′-trihydroxydihydrochalcone (**5**),[17] and uvangoletin (**6**).[18] The structures of isolated compounds are shown in Figure 1.

Panduratin C (**1**), $[\alpha]_D^{27}$: −24.0° (*c* 0.13, MeOH), was obtained as a yellow viscous oil and analyzed as $C_{26}H_{30}O_5$ ($[M]^+$ *m/z* 422.2044). The IR spectrum displayed absorption bands at 3438 (hydroxyl) and 1624 (conjugated carbonyl) cm^{-1}, and UV absorption bands at λ_{max} 220 and 292 nm supporting the presence of a conjugated carbonyl in the structure. The ^{13}C NMR and DEPT spectrum indicated the presence of 26 carbons as 12 aliphatic carbons (3Me, $2CH_2$, 3CH, and 2C=CH–), 12 aromatic carbons (6CH, 2C, and 4C–O), one carbonyl, and one methoxyl carbon. The ^1H NMR spectral data displayed a downfield resonance at δ 13.90, attributable to chelated hydroxyl group, while two doublets in the aromatic region (at δ 7.04 and 6.68, each 2H, J = 8.1 Hz) suggested the presence of a *para*-disubstituted aromatic ring. Two aromatic protons as two doublets at δ 5.89 and 5.92 (each J = 2.4 Hz) and one singlet at δ 3.90 were assigned to H-3, H-5, and OMe, respectively. The proton signals at δ 4.85 (1H, t, J = 6.6 Hz), 2.47 (1H, m), 2.26 (1H, m), and 1.50 (6H, s) indicated the presence of an isoprenyl moiety. Additionally, four methine proton signals at δ 5.42 (1H, br s, H-4′), 4.41 (1H, dd, J = 11.4, 4.5 Hz, H-1′), 3.35 (1H, td, J = 11.4, 6.6 Hz, H-6′), and 2.47 (1H, m, H-2′), and a vinylic methyl proton at δ 1.78 (3H, s) indicated that **1** had a cyclohexenyl chalcone skeleton.[14,15] The connectivity of H-4′/H-5′, H-5′/H-6′, H-6′/H-1′, H-1′/H-2′, H-2′/H-1″, and H-1″/H-2″ in COSY spectrum confirmed that isoprenyl group was connected to C-2′. In the HMBC spectrum, the methine proton at δ 4.41 (H-1′) correlated with carbons at δ 206.5 (C=O), 42.5 (C-2′), 35.8 (C-5′), 36.3 (C-6′), and 28.9 (C-1″), a methine proton at δ 2.47 (H-2′) with carbons at δ 124.2 (C-2″), 121.0 (C-4′), and 36.3 (C-6′), a methine proton at δ 3.35 (H-6′) with carbons at δ 139.2 (C-1‴), 128.1 (C-2‴/6‴), and 54.5 (C-1′), and methyl protons at δ 1.78 (3′-Me) with carbons at δ 137.2 (C-3′), 121.0 (C-4′), and 42.5 (C-2′). These evidences confirmed that the *para*-disubstituted aromatic ring, isoprenyl moiety, and vinylic methyl were attached to carbons C-6′, C-2′, and C-3′, respectively. The chelated hydroxyl group at δ 13.90 correlated with carbons at δ 167.5 (C-2), 106.8 (C-1), and 96.7 (C-3). The methoxyl proton at δ 3.90 was assigned at C-6 from its HMBC correlation (Fig. 2) with carbon at δ 162.8 (C-6) and a NOESY cross-peak with H-5 (δ 5.92). The relative stereochemistry of **1** was identified on the basis of coupling constants and NOESY experiments. The large J value of proton H-1′ (J = 11.4 Hz) indicated that H-1′ should be α-axial oriented.[14] In the NOESY, a methine proton at δ 4.41 (H-1′) showed cross-peaks with protons δ 2.47 (H-2′) and 7.04 (H-2‴/H-6‴) but none with proton at δ 3.35 (H-6′), suggesting that H-2′ and H-6′ should be α-equatorial and β-axial oriented, respectively. Thus, panduratin C was determined to be (2,4-dihydroxy-6-methoxyphenyl)[3′-methyl-2′-(3″-methylbut-2″-enyl)-6′-(4‴-hydroxyphenyl)cyclohex-3′-enyl]methanone (**1**).

The compounds (**1–6**) isolated from the rhizomes of *B. pandurata* were investigated for anti-HIV-1 PR activity. Cardamonin (**7**) used in this study was previously isolated from this plant by our group.[12] Among the isolated compounds tested, hydroxypanduratin A (**3**) exhibited the most potent HIV-1 PR inhibitory activity with an IC_{50} value of 5.6 μM, followed by panduratin A (**2**, IC_{50} = 18.7 μM), whereas other compounds possessed weak activity (Table 1 and Fig. 3). Structure–activity relationships of these class of compounds for anti-HIV-1 PR activity are summarized as follows: (1) hydroxyl moiety at position 4 conferred higher activity than the methoxyl group as observed in **3**

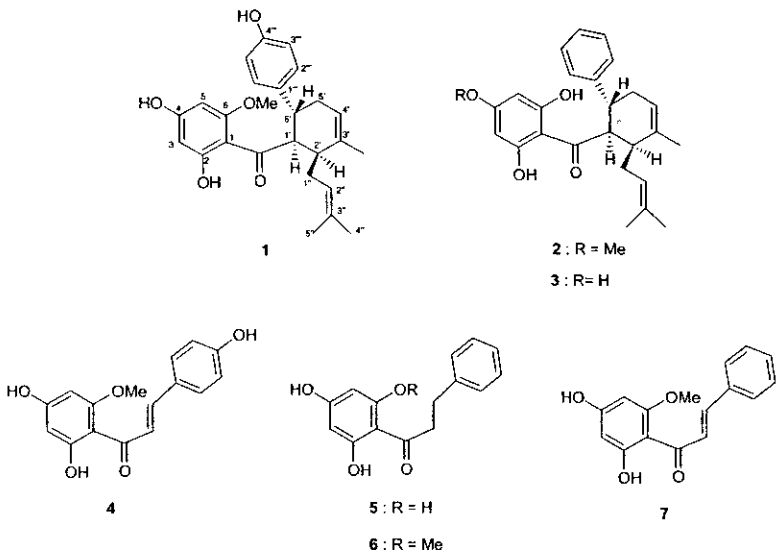

Figure 1. Chemical structures of compounds isolated from the rhizomes of *Boesenbergia pandurata*.

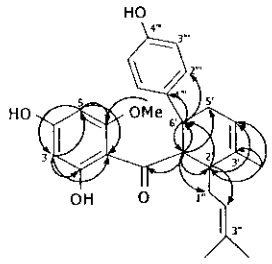

Figure 2. Selected HMBC correlations of panduratin C (1).

Table 1. HIV-1 PR inhibitory activity of compounds 1-7 of *Boesenbergia pandurata*[a], ( ) = % inhibition at 100 μM

Compound	IC_{50} (μM)
Panduratin C (1)	>100 (43.1%)
Panduratin A (2)	18.7 ± 0.8
Hydroxypanduratin A (3)	5.6 ± 0.7
Helichrysetin (4)	>100 (14.1%)
2′, 4′, 6′-Trihydroxyhydrochalcone (5)	>100 (7.5%)
Uvangoletin (6)	>100 (2.7%)
Cardamonin (7)	>100 (47.6%)
Acetyl pepstatin, positive control	3.4 ± 0.2

[a] Each value represents the mean ± SD of the three determinations.

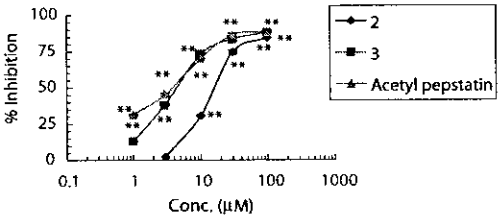

Figure 3. Dose–response curve of compounds 2 and 3 against HIV-1 PR comparing with acetyl pepstatin. Each value represents the mean ± SD of the three determinations. Significantly different from control: $^{*}p < 0.05$; $^{**}p < 0.01$.

(IC_{50} = 5.6 μM) versus **2** (IC_{50} = 18.7 μM); (2) prenylation of dihydrochalcone (**3**, IC_{50} = 5.6 μM) produced higher activity than non-prenylated one (**5**, IC_{50} > 100 μM); (3) hydroxylation at position 4‴ reduced activity, as observed in **7** (47.6%) versus **4** (14.1%); and (4) introduction of a double bond at C1′ and C6′ of chalcone gave higher activity as shown in **7** (47.6% inhibition) versus **6** (2.7% inhibition). In 1998, Ma and co-workers reported potent non-peptide HIV-1 PR inhibitors, ursolic acid and its glutaryl hemiester derivative, whose IC_{50} values are 8.0 and 4.0 μM, respectively.[19] These two compounds possessed comparable activity to hydroxypanduratin A (**3**, IC_{50} = 5.6 μM). The potency of **3** against HIV-1 PR was also comparable to that of acetyl pepstatin, a positive control (IC_{50} = 3.4 μM). Regarding bioactivities of constituents in *B. pandurata*, panduratin A (**2**) exhibited strong antibacterial activity against *Porphyromonas gingivalis*, a bacteria causing periodontitis.[20] This compound also possessed anti-inflammatory activity through inhibition of nitric oxide production induced by lipopolysaccharide (LPS) in RAW 264.7 cell line.[21] Moreover, Tuchinda and co-workers also reported a topical anti-inflammatory activity of hydroxypanduratin A (**3**) and panduratin A (**2**) on TPA-induced ear edema in rats.[15]

In conclusion, hydroxypanduratin A (**3**) and panduratin A (**2**) isolated from *B. pandurata* rhizomes are responsible for potent anti-HIV-1 PR activity. The structure–activity relationships of these compounds require the hydroxylation at position 4 and the prenylation of chalcone. This study also supports the use of *B. pandurata* by AIDS patients of Thailand.

3. Experimental

3.1. General experimental procedures

The optical rotation $[\alpha]_D$ values were determined with a JASCO P-1020 polarimeter. UV spectra were measured with a SPECORD S 100 (Analytikjena). The IR spectra were measured with a Perkin-Elmer FTS FT-IR spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded using a 300 MHz Bruker FT NMR Ultra Shield™ spectrometer. Chemical shifts were recorded in parts per million (δ) in $CDCl_3$ or CD_3OD with tetramethylsilane (TMS) as an internal reference. The EI-MS was obtained from a MAT 95 XL mass spectrometer. Quick column chromatography (QCC) and column chromatography (CC) were carried out on silica gel 60 F_{254} (Merck) and silica gel 100 (Merck), respectively. Precoated plates of silica gel 60 F_{254} and reversed phase (RP-18 F_{254S}) were used for analytical purposes.

3.2. Plant material

The fresh rhizomes of *B. pandurata* Holtt. were bought from Hat Yai Market, Hat Yai, Thailand. The voucher specimen (number: SN 4412015) was identified by Assoc. Prof. Dr. Sanan Subhadhirasakul and kept at the Herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

3.3. Extraction and isolation

Briefly, chopped-dried rhizomes (10.0 kg) of *B. pandurata* were extracted with $CHCl_3$ and MeOH (30 l × 3, 7 days each) at room temperature and the solvent was evaporated under reduced pressure to afford the $CHCl_3$ (608.40 g) and MeOH (211.70 g) extracts, respectively. A part of the MeOH extract (140 g) was further subjected to QCC on silica gel (200 g) eluting with hexane/CH_2Cl_2/MeOH (9:1:0, 1:1:0, 0:100:0, 0:19:1, 0:17:1, 0:1:1, and 0:0:100, each 1500 ml) to yield seven fractions (F1–F7). Fraction F2 (hexane/CH_2Cl_2, 1:1, 18.7 g) was chromatographed by QCC on silica gel (180 g) eluting with hexane/CH_2Cl_2 (1:1, 2000 ml) to give three subfractions (F2a–F2c). Subfraction F2c (1.03 g) was recrystallized from CH_2Cl_2 to give **2** (715.2 mg). Fraction F3 (CH_2Cl_2/MeOH, 19:1, 300 mg) was separated by CC on silica gel (18 g) with CH_2Cl_2/MeOH (19:1, 1000 ml) to afford four subfractions (F3a–F3d). Subfraction F3b (10.3 mg) was purified by preparative TLC with hexane/EtOAc (3:2) to obtain **4** (8.3 mg). Subfraction

F3c (130.0 mg) was separated by CC on silica gel (8 g) with hexane/EtOAc (13:7, 400 ml) to give **3** (36.6 mg) and **1** (6.2 mg). Fraction F4 (CH_2Cl_2/MeOH, 17:1, 1.2 g) was purified by CC on silica gel (60 g) and eluted with hexane/EtOAc (13:7, 1500 ml) to give four subfractions (F4a–F4d). Subfraction F4c (49.3 mg) was purified by reversed-phase preparative TLC with MeOH/H_2O (3:1) to afford **5** (25.2 mg). Subfraction F4d (898.0 mg) was subjected to CC on silica gel (60 g) with hexane/EtOAc (13:7, 1000 ml) to give **6** (21.0 mg).

3.3.1. Panduratin C (1). Yellow viscous oil; $[\alpha]_D^{27}$: −24.0° (*c* 0.13, MeOH); IR (neat): 3438, 1624 cm^{-1}; UV (MeOH): λ_{max} (log ε) = 292 (3.71), 220 (3.95) nm; ^1H NMR ($CDCl_3$, 300 MHz): δ 13.90 (1H, s, 2-OH), 7.04 (2H, d, J = 8.1 Hz, H-2‴/H-6‴), 6.68 (2H, d, J = 8.1 Hz, H-3‴/H-5‴), 5.92 (1H, d, J = 2.4 Hz, H-5), 5.89 (1H, d, J = 2.4 Hz, H-3), 5.42 (1H, br s, H-4′), 4.85 (1H, t, J = 6.6 Hz, H-2″), 4.41 (1H, dd, J = 11.4, 4.5 Hz, H-1′), 3.90 (3H, s, OMe), 3.35 (1H, td, J = 11.4, 6.6 Hz, H-6′), 2.47 (2H, m, H-2′, H-1″), 2.45 (1H, m, H-5′), 2.40 (1H, m, H-5′), 2.26 (1H, m, H-1″), 1.78 (3H, s, 3′-Me), 1.50 (6H, s, Me-4″/Me-5″); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 206.5 (C=O), 167.5 (C-2), 162.8 (C-6), 162.1 (C-4), 153.3 (C-4‴), 139.2 (C-1‴), 137.2 (C-3′), 131.8 (C-3″), 128.1 (C-2‴/C-6‴), 124.2 (C-2″), 121.0 (C-4′), 115.2 (C-3‴/C-5‴), 106.8 (C-1), 96.7 (C-3), 90.8 (C-5), 55.8 (OMe), 54.4 (C-1′), 42.5 (C-2′), 36.3 (C-6′), 35.8 (C-5′), 28.9 (C-1″), 25.6 (C-5″), 22.9 (3′-Me), 17.9 (C-4″); EI-MS: m/z = 422 [M^+] (2), 421 [M^+-1] (5), 406 (6), 286 (11), 166 (100), 106 (9); HR-MS: m/z = 422.2044 (calcd for $C_{26}H_{30}O_5$: 422.2088). Copies of original spectra are available from the author of correspondence.

3.4. Enzymes and chemicals

Recombinant HIV-1 PR, substrate peptides, and acetyl pepstatin were purchased from Sigma Chemical Co., St. Louis, USA.

3.5. Assay of HIV-1 protease inhibitory activity

This assay was modified from the previously reported method.[22] Briefly, the recombinant HIV-1 PR solution was diluted with a buffer composed of a solution containing 50 mM sodium acetate (pH 5.0), 1 mM ethylenediamine disodium (EDTA·2Na), and 2 mM 2-mercaptoethanol (2-ME), and mixed with glycerol in the ratio of 3:1. The substrate peptide, Arg-Val-Nle-(*p*NO_2-Phe)-Glu-Ala-Nle-NH_2, was diluted with a buffer solution of 50 mM sodium acetate (pH 5.0). Two microliters of plant extract and 4 μl of HIV-1 PR solution (0.025 mg/ml) were added to a solution containing 2 μl of 50 mM buffer solution (pH 5.0) and 2 μl of substrate solution (2 mg/ml), and the reaction mixture 10 μl was incubated at 37 °C for 1 h. A control reaction was performed under the same condition but without the plant extract. The reaction was stopped by heating the reaction mixture at 90 °C for 1 min. Subsequently, 20 μl of sterile water was added and an aliquot of 10 μl was analyzed by HPLC using RP-18 column (4.6 × 150 mm ID, Supelco 516 C-18-DB 5 μm, USA). Ten microliters of the reaction mixture was injected to the column and gradiently eluted with acetonitrile (15–40%) and 0.2% trifluoroacetic acid (TFA) in water, at a flow rate of 1.0 ml/min. The elution profile was monitored at 280 nm. The retention times of the substrate and *p*-NO_2-Phe-bearing hydrolysate were 11.356 and 9.457 min, respectively. The inhibitory activity on HIV-1 PR was calculated as follows: % inhibition = $(A_{control} - A_{sample}) \times 100/A_{control}$; whereas A is a relative peak area of the product hydrolysate. Acetyl pepstatin was used as a positive control.

3.6. Statistical analysis

For statistical analysis, the results of anti-HIV-1 PR activity were expressed as means ± SD of three determinations. The IC_{50} values were calculated using the Microsoft Excel program. Statistical significance was calculated by Dunnett's test.

Acknowledgments

We are grateful to the Thailand Research Fund and the Higher Education Development Project: Postgraduate Education and Research Program in Chemistry, the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0157/2547), Prince of Songkla University through Natural Products from Mangrove Plants and Synthetic Materials Research Unit (NSU) and Graduate School and the Royal Thai Government Budget for financial support.

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