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Isolation and characterization of aromatase inhibitors from *Brassaiopsis glomerulata* (Araliaceae)

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**Introduction**

*Brassaiopsis glomerulata* is a member of the Araliaceae that occurs in south and southeast Asia, including the Vietnamese peninsula (Van Kiem et al., 2003) and in Indonesia. *B. glomerulata* is a large shrub or small tree with thorns on the stems, palmate leaves with five to seven leaflets, pendulous panicles, and flowers in glomerulous heads (Regel, 1863). This species has several reported medicinal uses. In Vietnam, the plant is used to treat rheumatism and back pain (Van Kiem et al., 2003). In India, a group of indigenous tribes called the Nagas drink a juice extract of *B. glomerulata* bark to aid in digestion and to alleviate constipation. The Nagas also use a paste of the bark of *B. glomerulata* to treat bone fractures and sprains (Changkija, 1999). This species is also used medicinally in China as one of several kinds of "tongcao" (unlocking herbs used to promote urination and assist in lactation) (Shen et al., 1998). Owing to its use as a "tongcao", *B. glomerulata* was tested using *in vivo* anti inflammatory, anti pyretic, and diuretic models (Shen et al., 1998). Moreover, *B. glomerulata* is reported to inhibit carrageenan induced swelling of rat paws (anti inflammatory), and induce an antipyretic effect using a rat fever model induced by beer yeast or carrageenan, but no diuretic effect was reported (Shen et al., 1998).

Aromatase is the enzyme responsible for catalyzing the biosynthesis of estrogens (estrone and estradiol) from androgens (androstenedione and testosterone) (Johnston and Dowsett, 2003; Brueggemeier, 2006). Inhibition of aromatase has been shown to reduce estrogen production throughout the body to nearly undetectable levels and aromatase inhibitors are being used clinically to retard the development and progression of hormone responsive breast cancers [FDA approved aromatase inhibitors include anastrozole (Arimidex®), letrozole (Femara®) and exemestane (Aromasin®)]. As part of a research project directed
toward the study of new naturally occurring chemopreventive agents from plants (Kinghorn et al., 2004), the leaves of Brassaiopsis glomerulata (Blume) Regel (Araliaceae), collected in Indonesia, were found to inhibit the aromatase enzyme. Very limited phytochemical studies have been performed on plants in the genus Brassaiopsis, with the only isolates reported to date being three known triterpenes of the lupane subgroup (Van Kiem et al., 2003). Due to the strong aromatase activity and the lack of previous phytochemical research, bioassay guided fractionation of the leaves of B. glomerulata was initiated to isolate and identify compounds with potential aromatase inhibitory (AI) activity.

**Results and discussion**

A hexane soluble extract of the leaves of B. glomerulata exhibited significant aromatase inhibition with both enzyme based and cell based AI assays [6.9% control activity (PCA) at 20 μg/mL and 7.2 PCA at 20 μg/mL, respectively]. Bioassay guided fractionation of the hexane extract led to the isolation of six compounds of the steroid and triterpenoid classes [spinasterone (1) (Wandji et al., 2002), stigmasterol (2) (Forgo and Kover, 2004), spinasterol (3) (Kojima et al., 1990), 7β hydroxy 4,22 stigmasta dien 3 one (4) (Ayyad, 2002), 6β hydroxystigmasta 4 one (5) (Kontiza et al., 2006), and oleanolic acid (6) (Seebacher et al., 2003)] (Fig. 1). The ethyl acetate extract of B. glomerulata was also found to exhibit moderate aromatase inhibition with the enzyme based and cell based AI assays (59.3 PCA at 20 μg/mL and 37.0 PCA at 20 μg/mL, respectively).

Bioassay guided fractionation of the ethyl acetate extract led to the isolation of seven compounds of the dipeptide, modified peptide, fatty acid, monoterpenoid, and benzenoid classes [N benzoyl L phenylalaninyl N benzoyl L phenylalaninate (7) (Catalan et al., 2003), N acetyl L phenylalaninyl N benzoyl L phenylalaninate (8) (Xiao et al., 2002), N benzoyl L phenylalanine methyl ester (9) (Li et al., 2000), linoleic acid (10) (Ramsewak et al., 2001), ( ) dihydroactinidiolide (11) (Mori and Khlebnikov, 1993), ( ) dehydrololiolide (12) (Ravi et al., 1982), and 4 hydroxybenzalde hyde (13) (Levin and Du, 2002)] (Fig. 1).

Compound 9 was found to be a new natural product, not previously isolated from any organism. This compound was previously obtained as a synthetic peptide derivative, and was prepared using Lewis acid cleavage of a resin bond carbamate (Li et al., 2000). The stereochemistry of 9 at position C 8 was assigned tentatively as L based on the comparison of the observed [αD] of +75° with the literature (Li et al., 2000). Following hydrolysis, a Marfey's analysis was undertaken to confirm the stereochemistry at this position. Marfey's analysis has become one of the standard methods for the determination of absolute configuration of compounds containing modified amino acid residues (Lang et al., 2006; Mitova et al., 2006). In the region of interest of the HPLC chromatogram, a peak appeared corresponding to the L phe Marfey's derivative. To confirm this assignment, the Marfey's derivatives of L phe and D phe were co injected separately with the Marfey's derivative of the hydrolysate of 9. The L phe derivative directly overlapped the peak of the hydrolysate derivative, while the D phe derivative eluted considerably after the hydrolysate derivative. The stereochemistry of 9 at position C 8 was therefore determined to be L.

Marfey's analysis was also performed to confirm the stereochemistry of 7, with positions C 8 and C 8' both being found to have the L configuration, which was not determined at the time of its previous isolation (Catalan et al., 2003). Furthermore, the absolute stereochemistry of 8 at positions C 8 and C 8' was not assigned previously (Xiao et al., 2002). Using Marfey's analysis, the stereochemistry of positions C 8 and C 8' for compound 8 was determined to be L at both positions.

The fatty acid, linoleic acid (10), was found to be significantly more active than the positive control, aminoglutethimide (AG) in the enzyme based AI assay (7.4 PCA at 20 μg/mL) (P < 0.0001) (Fig. 1). However, linoleic acid (10) was inactive in the cell based assay (147.6 PCA at 100 μM, the interference of fatty acids in the non cellular, enzyme based radiometric AI assay was previously...
reported in Balunas et al., 2006]. Upon the isolation of linoleic acid from the ethyl acetate extract, the hexane extract was reexamined for the presence of this compound using comparative TLC patterns and trace amounts were found in the hexane extract also. Since unsaturated fatty acids can readily undergo lipid oxidation, forming hydroperoxides in the presence of oxygen (Boyd et al., 1992; Banni et al., 1996; Lee et al., 2005), it is possible that linoleic acid in the B. glomerulata extracts may have been oxidized during the course of bioassay guided fractionation, resulting in decreasing levels of activity of subsequent fractionation steps.

The monoterpenoid ( ) dehydroloiolide (12), was found to be active in the cell based AI bioassay (21.8 PCA at 50 μM) (Fig. 2), with no statistical difference in aromatase inhibition activity between compound 12 and the positive control, letrozole (P < 0.0001). The other monoterpenoid isolated from the ethyl acetate extract, ( ) dihydroactinidiolide (11), was not able to be tested in either AI assay due to compound volatilization. Monoterpenoids commonly undergo volatilization and are thus often used for flavoring or as perfume ingredients. The decreasing levels of activity of B. glomerulata during the course of bioassay guided fractionation may be the result of other active and similarly volatile monoterpenoids. Two chlorinated monoterpenoid pesticides, toxaphene and chlordane, have previously been reported to decrease aromatase activity in a SK BR 3 cell based AI assay, although the compounds were not direct inhibitors of aromatase but rather suppressed aromatase expression by antagonizing estrogen related receptor α1 (ERα1) (Yang and Chen, 1999; Chen et al., 2001). The difference in the activity of 12 in the enzyme based AI assay (91.5 PCA at 20 μg/mL) and in the cell based AI assay (21.8 PCA at 50 μM) may be the result of indirect modulation of aromatase activity as was found with these chlorinated monoterpenoid pesticides.

The new natural product, N benzoyl L phenylalanine methyl ester, 9, was also found to be moderately active in the cell based AI bioassay (33.3 PCA at 50 μM), although 9 was not active in the enzyme based AI assay (94.3 PCA at 20 μg/mL). Some structural similarities exist between the chemical structure of 9 and letrozole, one of the AIs currently in clinical use, with two benzyl rings separated by an alkyl linker that contains a nitrogen. However, a detailed investigation of the structural similarities of 9 as compared with anastrozole and letrozole was not possible during the course of the present study.

6β Hydroxystigmasta 4 en 3 one (5) was found to be weakly active in the cell based AI bioassay. Interestingly, compound 5, as well as compounds 9 and 12, were not active in the enzyme based AI assay, perhaps indicating that they are acting through indirect regulation or modulation of aromatase activity rather than through direct aromatase inhibition (Yang and Chen, 1999; Chen et al., 2001; Díaz Cruz et al., 2005).

Brassaiopsis glomerulata has been reported to have various ethnobotanical uses, as mentioned earlier. The past history of medicinal use by human populations of B. glomerulata may be indicative of being safe when consumed. The strong aromatase inhibition of the hexane extract of B. glomerulata in both enzyme and cell based assays, coupled with the possibility of a favorable safety profile, may point to the potential for use of B. glomerulata for the chemoprevention of breast cancer. Further investigations of B. glomerulata are needed, including a recollection and subsequent bioassay guided compound isolation, as well as further biological studies on the mechanism of aromatase inhibition and in vivo testing.

Experimental

General experimental procedures

Enantioselectively pure standards L phenylalaninol (L phe ol), d phenyl alaninol (D phe ol), L phenylalanine (L phe), and D phenyl alanine (D phe) were purchased from Sigma Aldrich (St. Louis, MO). Na2 (2.4 Dinitro 5 fluorophenyl) L alaninamide (D FDA, Marfey’s reagent) was purchased from Sigma Aldrich (St. Louis, MO). HPLC of Marfey’s derivatives was performed using two Waters 515 pumps and a Waters 2487 dual wavelength detector (Waters, Milford, MA). A preparative Waters SunFire™ C18 column (19 × 150 mm) was used for fractionation, while an analytical Waters SunFire™ C18 column (4.6 mm × 150 mm) was used for Marfey’s analysis. Solvents were HPLC grade and used without further purification. Radiolabeled [1β 3H]androst 4 ene 3,17 dione, scintillation cocktail 3a70B, and SK BR 3 human breast cancer cells were obtained as described previously (Brueggemeier et al., 2005). Radioactivity was counted on an LS6800 liquid scintillation counter (Beckman, Palo Alto, CA). All other chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO).

Plant material

The leaves of Brassaiopsis glomerulata (Blume) Regel were collected by Dr. Soedarsono Riswan, Herbarium Bogoriense, Bogor, Indonesia, in 1996 (collection number BK 35). Dried plant material was stored at ambient temperature at the UIC Pharmacognosy Field Station in Downers Grove, Illinois until used for the present study. A voucher specimen (accession number P1750) has been deposited at the Field Museum of Natural History, Chicago, Illinois.

Extraction and isolation

Air dried leaves of B. glomerulata (1.1 kg) were ground and extracted with methanol overnight (3 × 3 L). The macerate was concentrated in vacuo (46.1 g) and partitioned to afford a petroleum ether extract (11.6 g), an ethyl acetate extract (8.2 g), and an aqueous extract (13.3 g).

The petroleum ether extract was fractionated using Si gel vacuum liquid chromatography (Alrich, Si gel 60, 63 200 mesh, 8.5 × 18 cm) using 100% petroleum ether, followed by a gradient of increasing polarity of petroleum ether ethyl acetate, followed, in

![Fig. 2. Percent control activity (PCA) at 20 μg/mL for compounds 1–13 isolated from Brassaiopsis glomerulata in the non-cellular, enzyme-based aromatase bioassay and in SK-BR-3 hormone-independent human breast cancer cells that overexpress aromatase (DMSO = dimethyl sulfoxide, blank/negative control; AG = aromatase inhibitor, positive control in non-cellular assay, 50 μM; LET = letrozole, positive control in cell-based assay, 10 nM). Each bar represents the mean and standard error of three replicates, with statistically significant differences indicated in the non-cellular assay by different numbers of stars and in the cell-based assay by different letters (P < 0.0001).](image-url)
turn, by ethyl acetate methanol. The column was then washed with 100% methanol. Altogether, 10 pooled fractions (F005 F014) were collected. Compound 1 (3.6 mg) was obtained as a precipitate from F006. A white solid precipitated from fraction F007, a mixture of two related compounds, and was chromatographed using silica gel (Aldrich, Sil gel 60, 230 400 mesh, 4.5 46 cm), beginning with 8:1 hexane ethyl acetate, followed by a gradient of increasing polarity, and washed with 100% methanol to afford purified stigmastanol (2, 2.2 mg) and purified spinasterol (3, 1.9 mg).

Fractions F008, F009, and F010 were all considered active in the non cellular assay and were combined based on similar TLC profiles (for a total of 3.1 g). F008 F010 was fractionated using Diaion HP 20 sorbent (Aldrich, 4.0 cm, 1.0 mL) eluted with 1:1 MeOH H2O, 3:1 MeOH H2O, 100% MeOH, 3:1 MeOH acetone, and 1:1 MeOH acetone, and 100% acetone. Compound 6 (2.7 mg) was obtained as a white precipitate from the 100% MeOH fraction. The 3:1 MeOH acetone fraction (1.9 g) was further fractionated (Aldrich, Sil gel 60, 230 400 mesh, 2.5 45 cm), starting with 9:1 hexane acetone and continuing with increasing polarity until washing with 100% acetone, followed by 100% methanol. Five pooled fractions were obtained with the 3:2 hexane acetone fraction (0.09 g) being further fractionated by preparative reversed phase HPLC (92% methanol in water, 8 mL/min, monitoring at 238 and 275 nm), yielding 4 (1.1 mg, tf 36.8 min) and 5 (0.4 mg, tf 50.4 min).

The ethyl acetate extract (8.1 g) was fractionated using Si gel vacuum liquid chromatography (Aldrich, Sil gel 60, 63 200 mesh, 8.5 19 cm), beginning with 100% petroleum ether, followed by a gradient of petroleum ether ethyl acetate, ethyl acetate methanol, and 100% methanol. Altogether 17 pooled fractions (F029 F045) were collected. Compound 7 (19.8 mg) precipitated from fraction F034. F031 (0.01 g) was subjected to further fractionation (Aldrich, Sil gel 60, 230 400 mesh, 1.0 15 cm) using an isocratic 5:1 hexane ethyl acetate system, yielding pure compound 10 (0.1 mg). Fractions F032 and F033 were combined (0.1 g) and fractionated by preparative reversed phase HPLC separations (60% water in acetonitrile, 7 mL/min, monitoring at 220 and 254 nm) to afford 11 (0.9 mg, tf 11.5 min).

Fraction F034 (0.3 g) was chromatographed using Sephadex LH 20 gel (Aldrich, 2.5 76 cm) in methanol, to afford 12 pooled fractions (F055 F066). The combination of F057 F058 (0.1 g) was worked up by preparative reversed phase HPLC separations (7 mL/min, monitoring at 220 and 254 nm). The solvent conditions for separation involved a 85:15 water acetonitrile system for 10 min followed by a 60 min gradient to 1:9 water acetonitrile, to afford compounds 12 (3.5 mg, tf 29.5 min), 9 (0.8 mg, tf 46.1 min), and 8 (3.7 mg, tf 50.8 min). Fractions F059 and F060 were combined (0.02 g) and separated using preparative reversed phase HPLC separations (7 mL/min, monitoring at 220 and 254 nm). The solvent conditions for separation involved an 85:15 water acetonitrile system for 15 min followed by a 45 min gradient to 1:9 water acetonitrile, to afford compound 13 (1.9 mg, tf 24.2 min).

**Marfey's analysis**

Samples of 7 9 were independently hydrolyzed at 110 °C with 6N HCl for 1 h (Fujii et al., 2002). Hydrolysates were dried and Marfey's derivatives were prepared following a published procedure (Fujii et al., 2002). The dried hydrolysates of 7 9, as well as the standards L phe, D phe, L o-lphe, D o-lphe, and D L-phenylalanine, were each dissolved in 100 mL of water. A 1 M sodium bicarbonate solution was prepared and 40 mL was added to each sample. A 1% (w/v) solution of L FDAA in acetonitrile was prepared and 100 mL was added to each vial. Each vial was then vortexed and incubated at 40 °C for 1 h. To quench reactions, 40 mL of 1N HCl were added. Each of the Marfey's derivatives of the hydrolysates and standards were separately analyzed by HPLC. All four Marfey's derivatives of the standards were then analyzed concurrently. Each Marfey's derivative of the hydrolysates was then analyzed concurrently with each Marfey's derivative of the standards. The reaction mixtures were analyzed by HPLC using a 5 min isocratic mobile phase of 30% CH3CN/70% 0.01N aqueous TFA followed by a gradient elution profile to 90% CH3CN/10% 0.01N aqueous TFA over 60 min at a flow of 1 mL/min, monitoring at 340 and 256 nm.

**Non cellular, enzyme based aromatase bioassay**

This assay was performed as described in earlier publications (Kellis and Vickery, 1987; O'Reilly et al., 1995; Balunas et al., 2006). Human placental microsomes were obtained from human term placentas that were processed at 4 °C immediately after delivery from The Ohio State University Medical Center [OSU Institutional Review Board (IRB) protocol number 2002H0105, last approved in December 2006]. Extracts and compounds were originally screened at 20 µg/mL in DMSO using a non cellular microbial radiometric aromatase assay. Samples [extracts or compounds, DMSO as negative control, or 50 µM (+) aminogluthethimide (AG) as positive control] were tested in triplicate. Each reaction mixture included sample, 100 nM [1H]androst 4 ene 3 17dione (400,000, 450,000 dpm), 0.1 M potassium phosphate buffer (pH 7.0), 5% propylene glycol, and an NADPH regenerating system (containing 2.85 mM glucose 6 phosphate, 1.8 mM NADP+, and 1.5 units glucose 6 phosphate dehydrogenase). Microsomal aromatase (50 µg) was added to initiate the reactions, which were then incubated in a shaking water bath at 37 °C, and quenched after 15 min using 2 mL CHCl3. An aliquot of the aqueous layer was then added to 370B scintillation cocktail for quantitation of the formation of 3H2O. Percent control activity (PCA) was determined as previously described (Kellis and Vickery, 1987; O'Reilly et al., 1995; Balunas et al., 2006).

**Cell based aromatase bioassay**

Samples found to be active using the non cellular assay were further tested at various concentrations in SK BR 3 human breast cancer cells that overexpress aromatase, using a previously described method (Natarajan et al., 1994; Richards and Bruegge meier, 2003; Balunas et al., 2006). Cells were treated in triplicate with samples or 0.1% DMSO (negative control) or 10 nM letrozole (positive control). Results are initially determined as picomoles of 3H2O formed per hour incubation per million live cells (pmol/h/106 cells), with PCA calculated by comparison with the negative control, DMSO.

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