Anti-androgenic activity of furanodiene isolated from *Curcuma aeruginosa* Roxb. extract

Jukkarin Srivilai[a], Nantaka Khorana[a], Neti Waranuch[b] and Kornkanok Ingkaninan[a*]

[a]Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Science and Center of Excellence for Innovation in Chemistry, Naresuan University, Phitsanulok, Thailand

[b]Department of Pharmaceutical Technology, Faculty of Pharmaceutical Science and Center of Excellence for Innovation in Chemistry, Naresuan University, Phitsanulok, Thailand

*Corresponding Author. E-mail address: k_ingkaninan@yahoo.com (K. Ingkaninan)

Received 30 May 2011; accepted 31 August 2011

บทคัดย่อ

Human 5-alpha reductase enzyme is involved in the development of benign prostatic hyperplasia, androgenic alopecia, hirsutism, prostate cancer, and acne skin. The inhibitors of this enzyme can be used for the treatment of these diseases. From our previous *vitro* and *in vivo* studies, the rhizome of a Thai medicinal plant, *Curcuma aeruginosa* Roxb. (Zingiberaceae) showed significant anti-androgenic effects. In this study, we aim to search for the 5-alpha reductase inhibitors from the plant. The major constituent in this plant, furanodiene was isolated and identified using chromatographic and spectroscopic techniques. At the concentration of 1 mg/ml, furanodiene showed 40% inhibition effect on 5-alpha reductase. This sesquiterpenenoid compound can be a potential lead for the development of anti-androgenic drugs.

Keywords: anti-androgen, 5-alpha reductase inhibitor, *Curcuma aeruginosa* Roxb., furanodiene, sesquiterpenes

Introduction

In many tissues including of the prostate, skin and liver, testosterone is metabolized intracellularly by the enzyme testosterone 5-alpha reductase to an active androgen, 5-alpha dihydrotestosterone, which has high affinity binding to androgen receptors and shows various hormonal actions. Androgens have been indicated to be factors inciting common baldness (androgenic alopecia), benign prostatic hyperplasia (BPH), prostate cancer, acne skin, and, hirsutism (Russell & Willson, 1994). Thus, the anti-androgens which exhibit inhibitory activity on 5α-reductase and/or androgen receptor may be useful for treatment of these androgen-dependent disorders. Finasteride has been approved by the U.S. Food and Drug Administration (FDA) since 1992 for BPH treatment. In addition, this group of drugs is used to treat male pattern baldness. However, finasteride was reported to cause possible adverse effects; gynecomastia, impairment muscle growth and severe myopathy (Gormley, 1995). Therefore, the search for new anti-androgenic compounds is the great demand. Natural products have been considered as the potential sources of drugs (Aggarwala et al., 2010).

In the previous study, we found that the hexane extract from the rhizomes of a Thai medicinal plant, *Curcuma aeruginosa* Roxb. (Zingiberaceae) showed high anti-androgenic activity both *in vitro* and *in vivo* (Pumthong, 2010). *C. aeruginosa* is commonly found throughout the Southeast Asia. It is known in Thai as "Wan-Ma-Ha-Mek" and "Kha-min-dam". The colour of fresh rhizome can be yellow, green and blue. It has the ginger-like aroma (Sharad et al., 2006). The rhizomes of this plant have been used in traditional medicine for gastrointestinal and uterine remedies. Various biological activities of *C. aeruginosa* have been reported such as poscoital contraceptive effect, anti-HIV, hepatoprotective, antimicrobial, antioxidant, antiplatelet-activating factor and antinociceptive (Thaina et al., 2009; Otake et al., 1995; Trakoontivakorn et al., 2000). Sesquiterpenes have been found as common chemical constituents of this plant. The samples of sesquiterpenes in this plant are zedoalactone A, zedoalactone B, isofuranodiene, furanodiene, furanodienone, dehydrocurdione, curcumenone, 13-hydroxygermacrone, zedoarol, zedoarondiol, curcumenol, isocurcumenol, 1, 8-cineole, curzerenone, furanogermenone, camphor, (Z)-3-hexenol, furanodienone, beta-elemene, curzerene and germacrone (Takano et al., 1995; Kitamura et al., 2007; Sukari et al., 2007).

The purpose of this study was to isolate and identify the anti-androgenic compounds from the rhizomes of *C. aeruginosa* using bioassay-guided fractionation

technique. The anti-androgenic activity of the isolated compound was investigated on *in vitro* enzymatic assay.

Materials and Methods

General experimental procedures

The UV spectrum was obtained on a Shimadzu UV-2410 PC spectrophotometer. GC-MS analyses were conducted on Hewlett Packard 5989MS Engine model and HP-5MS capillary column (split ratio. 50:1). The following programs were used: GC condition; initial temperature 50°C for 5 min, ramp 10°C/min to 100 °C at an injection temperature of 100°C, MS detector was EIMS for analysis. The ^{1}H NMR and ^{13}C NMR were recorded on a Bruker Avance 400 NMR spectrometer at 400 and 100 MHz, respectively and NMR spectra were recorded in $CDCl_3$ solvent. The silica gel used for quick column chromatography (Merck 60, 230-400 mesh). TLC was performed with Kieselgel 60F-254 plate with 0.25 mm thickness. Visualizations of the TLC spots were performed by spraying with anisaldehyde and/or UV light 254 and 366 nm.

Plant materials and extraction

Fresh rhizomes of *C. aeruginosa* Roxb. (5 kg) were collected from Amphor Mueng, Phitsanulok province, Thailand. The plant materials were dried and powdered. The dried powder rhizomes (1.5 kg) were extracted with hexane (6L) for 3 days at room temperature and filtered. The maceration procedure was repeated 3 times. The solvent was evaporated under reduced pressure to afford crude hexane extract of *C. aeruginosa*. (30.01 g, 0.6% yield)

Isolation of furanodiene

The hexane extract (34.58 g) was fractionated by a quick column chromatography (Column: 10x13 cm) with gradient elution of hexane to ethyl acetate to provide 11 fractions. Fraction 1 was obtained as crude oil (5.85 g; 17% yield) and kept at 4°C to give colorless crystals. They were recrystalized with methanol twice to obtain furanodiene 4.32 g, (12.5% yield).

Furanodiene: TLC Rf 0.6 (n-heptane) quenching under UV 254 nm and positive with anisaldehyde to orange spot; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.967 (^{1}H, dd, *J* = 6.9, 10.8 Hz, H-1), 3.13 (2H, brd, *J* = 6.9, H-2α, H-2β), 2.12-2.18 (2H, m, , H-3α, H-3β), 4.77(1H, brt, *J* = 7.3, H-5), 1.83 (1H, dt, *J* = 7.3, 11.7, H-6α), 2.88 (1H, td, *J* = 11.7, 3.2, H-6β), 3.58 (1H, d, *J* = 14.4, H-9α), 3.47 (1H, d, *J* = 14.4, H-9β), 7.09 (1H, s, H-12), 1.95 (3H, s, H-13), 1.64 (3H, s, H-14), 1.31(3H,s, H-15); ^{13}C NMR ($CDCl_3$) 100 MHz δ: 129.02 (C-1), 24.38 (C-2), 26.86 (C-3), 128.76 (C-4), 127.66 (C-5), 39.52 (C-6), 118.86 (C-7), 149.71 (C-8), 40.94 (C-9), 134.35 (C-10), 121.83 (C-11), 136.01(C-12), 8.91 (C-13), 16.46 (C-14), 16.22 (C-15), GC-EIMS; GC retention time: 49.04 min, EIMS *m/z* 216 $[M]^+$ ($C_{15}H_{20}O$).

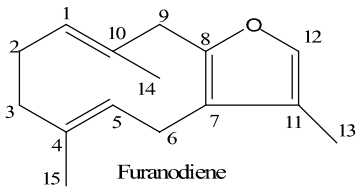
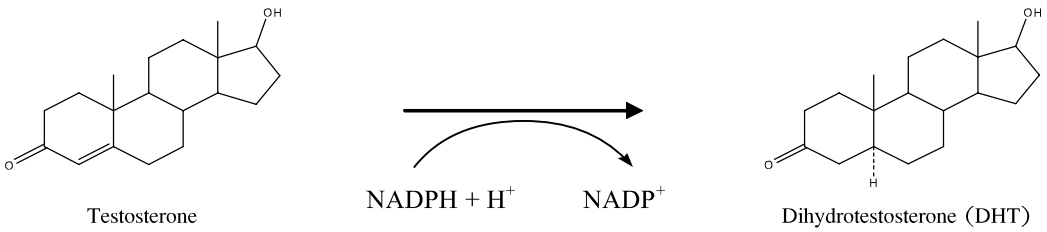


Figure 1. Structure of furanodiene

Preparation of rat liver microsomes

Rat livers of male sprague dawley (SD) rats (6 weeks of age) were used as sources of enzyme (Matsuda et al., 2001a,b). After the rats were sacrificed, the livers were removed and washed with Krebs-ringer phosphate buffer pH 7.4. The livers were homogenized in Tris-HCl buffer pH 7.4. The homogenate was then centrifuged at 6172, 193199 and 697291 g, respectively. In each time, the supernatant was collected and was centrifuged again at next speed. The pooled supernatant was collected at -80 C just before used.

Measurement of inhibitory activity against conversion of testosterone



Testosterone Dihydrotestosterone (DHT)

Figure 2. The enzymatic reaction catalyzed by steroid 5α-reductase *(Hiipakka & Liao, 1998)*

The *in vitro* anti androgen assay mimicked enzymatic reaction presented in Figure 2. The inhibitory activity against the conversion of testosterone to DHT of the samples was tested *in vitro* using rat liver enzyme in comparison with a positive control, ethynylestradiol. The decrease of testosterone after the enzymatic reaction was determined using an HPLC (Matsuda et al, 2001a, b). Briefly, the test solution contained 50 µL of test compound dissolved in DMSO, 350 µLof Tris-buffer pH 7.2, 100 µL of testosterone (0.5 mg/mL in the 1:1 v/v mixture of propylene glycol and Tris-buffer pH 7.2) and 350 µL of enzyme. The reaction was started by addition of 150 µL of NADPH (5.13 mg/mL in Tris-buffer pH 7.2). The mixture was incubated at 37°C for 30 min and reaction was stopped by addition of 3 mL of CH_2Cl_2. After that, 150 µL of internal standard (IS), prednisolone (0.1 mg/mL in methanol) was added. The tube was shaken and centrifuged at 3000 rpm (10 min, 4°C). The organic layer was transferred to another tube and evaporated to dryness. The residue was dissolved in 2 mL of methanol.

The test samples were separated into 3 groups; (1) $control_{0\ min}$, (2) $control_{30\ min}$, and (3) test sample (test compound or positive control). In the $control_{0\ min}$ tube, 3 mL of dichloromethane was added before the addition of NADPH, while in $control_{30\ min}$ tube, the test sample was replaced by 50µL of DMSO. The remained testosterone was determined by HPLC. The HPLC separation was conducted at 40°C on a Phenomenex Luna 5u C-18 column. Methanol:water (65:35 v/v) was used as a mobile phase. The flow rate of 0.8 mL/min was used. The injection volume was 20 µL. The detection was set at 254 nm. The percent enzymatic inhibition was determined using peak height ratios (r = peak height of testosterone / peak height of IS) as shown below:

$$\text{The percentage of ezymatic inhibition} = \frac{\text{r of test sample} - \text{r of test control}_{30\ min}}{\text{r of test control}_{0\ min}} \times 100$$

Results and Discussion

The hexane extract of *C. aeruginosa* was fractionated using chromatographic and recrytallization techniques to give a sesquiterpene, furanodiene. Its structure was elucidated using NMR techniques and the data was in agreement with previous report (Makabe et al., 2006). To examine the *in vitro* anti-androgenic activity of the samples, the inhibitory activity against the conversion of testosterone to DHT by 5α-reductase prepared from the rat liver enzyme was evaluated. HPLC was used to detect testosterone remaining after the enzymatic reaction. Percent enzymatic inhibition of the hexane extract of *C. aeruginosa* and furanodiene at the concentrations of 1 mg/mL was measured. Ethynylestradiol (1 mM) was used as a reference compound. The results are shown in Table 1. Furanodiene showed significant inhibitory activity (40.67%). The androgenic activity of this compound has never been reported before. Previously, furanodiene has been reported for anti-inflammatory effect and protective effect on liver injury induced by D-galactosamine/ lipopolysaccharide (Matsuda et al., 1998; Morikawa et al., 2002). It could also induce G2/M cell cycle arrest and apoptosis through MAPK signaling and mitochondria-caspase pathway in human hepatocellular carcinoma cells (Xiao et al., 2007). The inhibitive effects on cell proliferation were studied in several cell lines and the cytotoxic effects of furanodiene against HeLa, Hep-2, HL-60, U251 cells were observed after 12 h of administration. Furanodiene was also inhibited on the proliferation of uterine cervix (U14) tumor induced in mice (Zhen-zhen et al., 2009). The anticancer activity of furanodiene was also reported by Ying et al (2010).

From our study, it is noted that the hexane extract of *C. aeruginosa* showed higher enzymatic inhibitory activity than furanodiene. Thus, the extract may contain more than one compound responsible for anti-androgen activity.

Table 1. The percent enzymatic inhibition and IC_{50} values of *C. aeruginosa* extract and furanodiene at the final concentration of 1.0 mg/mL. The data are expressed as means ±SD.

Compounds	Enzymatic inhibition (%)	IC_{50} (mg/mL)
C. aeruginosa extract	72.78 ± 2.60	0.22 ± 0.03
Furanodiene	40.67 ± 4.23	Not detected
Ethynylestradiol	47.61 ± 5.49*	0.26 ± 0.02

Remark: * tested at the final concentration of 1 mM

Conclusion

From the bioassay guided fractionation of the hexane extract of *C. aeruginosa* rhizomes, a sesquiterpene, furanodiene was obtained. It showed inhibitory activity on 5-alpha reductase. This compound is a potential lead for new anti-androgens.

Acknowledgments

The Center for Innovation in Chemistry (PERCH CIC), Commission on Higher Education, Ministry of Education and Naresuan University are gratefully acknowledged.

References

Aggarwal, S., Thareja, S., Verma, A., Bhardwaj, T. R. & Kumar, M. (2010). An overview on 5alpha-reductase inhibitors. *Steroids*, 75, 109-153.

Gormley, G. J. (1995). Finasteride: a clinical review. *Biomedicine & Pharmacotherapy*, 49, 319-324.

Hiipakka, R. A. & Liao, S. (1998). Molecular mechanism of androgen action. *Trends in Endocrinology and Metabolism*, 9, 317-324.

Kitamura, C., Nagoe, T., Prana, M., Agusta, A., Ohashi, K. & Shibuya, H. (2007). Comparison of *Curcuma sp.* in Yakushima with *C. aeruginosa* and *C. zedoaria* in Java by trn K gene sequence, RAPD pattern and essential oil component. *Journal of Natural Medicines*, 61, 239-243.

Makabe, H., Maru, N., Kuwabara, A., Kamo, T. & Hirota, M. (2006). Anti-inflammatory sesquiterpenes from *Curcuma zedoaria*. *Natural Product Research: Formerly Natural Product Letters*, 20(7), 680 - 685.

Matsuda, H., Ninomiya, K., Morikawa, T. & Yoshikawa, M. (1998). Inhibitory effect and action mechanism of sesquiterpenes from zedoariae rhizoma on D-Galactosamine/ lipopolysaccharideinduced liver injury. *Bioorganic & Medicinal Chemistry Letters*, 8, 339-44.

Matsuda, H., Sato, N., Yamazaki, M., Naruto, S. & Kubo, M. (2001a). Testosterone 5alpha-reductase inhibitory active constituents from Anemarrhenae rhizoma. *Biological & Pharmaceutical Bulletin*, 24, 586-587.

Matsuda, H., Yamazaki, M., Matsuo, K., Asanuma, Y. & Kubo, M. (2001b). Anti-androgenic activity of Myricae Cortex-isolation of active constituents from bark of *Myrica rubra*. *Biological & Pharmaceutical Bulletin*, 24, 259-263.

Morikawa, T., Matsuda, H., Ninomiya, K. & Yoshikawa, M. (2002). Medicinal food stuffs. XXIX. Potent protective effects of sesquiterpenes and curcumin from Zedoariae rhizoma on liver injury induced by D galactosamine/lipopolysaccharide or tumor necrosis factor-alpha. *Biological & Pharmaceutical Bulletin*, 25, 627-631.

Otake, T., Mori, H., Morimoto, M., Ueba, N., Sutardjo, S., Kusumoto, I. T., Hattor, M., Namba, T. (1995). Screening of Indonesian plant extracts for anti-human immunodeficiency virus-type 1 (HIV-1) activity. *Phytotherapy Research*, 9, 6-10.

Pumthong, G. (2010). *The study on anti-androgen activity of Curcuma aeruginosa* Roxb. Thesis, Naresuan University at Phitsanulok.

Russell, D. W. & Wilson, J. D. (1994). Steroid 5alpha-Reductase: Two Genes/two enzymes. *Annual Review of Biochemistry*, 63, 25-61.

Sharad, S., Nitin, Ch., Sanjay, S., Mathew, D. AKS R. & Palpu, P. (2006). Pharmacognostic evaluation of *Curcuma aeruginosa* Roxb. *Natural Product Science*, *12*(3), 162-165.

Sukari, M. A. H., Saad, S. M., Lajis, N. H., Rahmani, M., Muse, R., Yusuf, U. K. & Riyanto, S. (2007). Chemical constituents and bioactivity of *Curcuma aeruginosa* Roxb.. *Natural Product Science*, 13, 175-179.

Takano, I., Yasuda, I., Takeya, K. & Itokawa, H. (1995). Guaiane sesquiterpene lactones from *Curcuma aeruginosa*. *Phytochemistry*, 40, 1197-1200.

Thaina, P., Tungcharoen, P., Wongnawa, M., Reanmongkol, W. & Subhadhirasakul, S. (2009) Uterine relaxant effects of *Curcuma aeruginosa* Roxb. rhizome extracts. *Journal of Ethnopharmacology*, 121, 433-443.

Trakoontivakorn G. & Saksitpitak J. (2000). Antioxidative potential of Thai indigenous vegetable extracts. *Institute of Food and Research and Product development, Kasetsart,* 30, 164-176.

Xiao Y., Yang F. Q. & Li S.P. (2007). Furanodiene induces G2/M cell cycle arrest and apoptosis through MAPK signaling and mitochondria-caspase pathway in human hepatocellular carcinoma cells. *Cancer Biology & Therapy*, 6, 1044-50.

Ying, W. L., Guo, Y. Z., Xiao, L. Sh., Jian, H. Ch., Zhi, L. Y. & Wang, F. F. (2010). Furanodienone induces cell cycle arrest and apoptosisby suppressing EGFR/HER2 signaling in HER2-overexpressing human breast cancer cells. *Cancer Chemotherapy & Pharmacology*, 00280-011-1624.

Zhen-zhen, B., Yan-ping, Zh., Hui, Zh., Xiu-yan, S. & Dong-hai, L. (2009). Potential Anti-cancer Activity of Furanodiene. *Anticancer Pharmacology Chinese Journal of Cancer Research, 21*(2), 154-158.