— Research Letter —

Abietic Acid from Resina Pini of *Pinus* Species as a Testosterone 5α-Reductase Inhibitor

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The 50% ethanol extracts from Resina Pini of *Pinus* sp. (Pinaceae) showed more potent inhibitory activity against testosterone 5α-reductase prepared from rat prostate than those from several medicinal plants used for the treatment of androgen-dependent diseases such as benign prostatic hyperplasia. The fraction responsible for this activity was purified, and the active constituent was isolated and identified as abietic acid, a diterpene resin acid, which exhibited potent testosterone 5α-reductase inhibitory activity. Methyl abietate was substantially inactive against testosterone 5α-reductase, whereas other diterpene resin acids, pimaric acid and neoabietic acid, were as active as abietic acid against testosterone 5α-reductase, indicating that the negatively charged anionic carboxyl group on the molecule is an important structural moiety for the inhibitory activity. These findings suggest that a nonsteroidal anionic diterpene compound of natural origin may have the potential to act as a transition state analogue inhibitor of testosterone 5α-reductase in the treatment of androgen-dependent diseases.

Key words —— abietic acid, Resina Pini, testosterone 5α-reductase inhibitor, resin acid, *Pinus* sp., androgen-dependent disease

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INTRODUCTION

Testosterone 5α-reductase [reduced nicotinamide adenine dinucleotide phosphate: Δ^4-3-ketosteroid 5α-oxidoreductase: Enzyme Commission (EC) 1.3.99.5] catalyzes the reductive conversion of testosterone, the major circulating androgen, to 5α-dihydrotestosterone in target tissues such as the prostate gland and nongenital skin. Testosterone 5α-reductase exists at least as type 1 and type 2 isozymes in the human and the rat.[1] It is generally believed that androgen-dependent diseases such as benign prostatic hyperplasia, male pattern baldness, and acne are associated with an overproduction of 5α-dihydrotestosterone in many tissues.[1] Therefore inhibition of this enzymatic conversion represents an important pharmacologic approach to the selective treatment of these androgen-dependent diseases.[2] Most testosterone 5α-reductase inhibitors developed are steroid derivatives, which bind to steroid receptors and are expected to produce various undesirable hormonal effects by acting as agonists or antagonists.[2] Alternatively, phytotherapeutic preparations from many medicinal plant sources have been commonly used for the treatment of such androgen-dependent diseases.[3] In particular, extracts from several types of medicinal plant sources have remarkable blocking effects on testosterone 5α-reductase activity.[3] Moreover, specific nonsteroidal constituents with moderately potent testosterone 5α-reductase inhibitory activity have been isolated from several selected medicinal plant species.[4–10]

In the course of examining the effects of several medicinal plants used as integrated medicines against androgen-dependent diseases on the activity of testosterone 5α-reductase prepared from rat prostate, we found that the 50% ethanol extract of Resina Pini of *Pinus* sp. (Pinaceae) showed potent inhibitory activity. In the present study, we investigated the active components in the 50% ethanol extract of Resina Pini of *Pinus* sp. to determine their testosterone 5α-reductase inhibitory activity against androgen-dependent diseases.

MATERIALS AND METHODS

General Experimental Procedures —— MS with the electron impact of 70 eV were obtained

with a JEOL JMS AX505WA mass spectrometer. ^{1}H- and ^{13}C-NMR spectra at 400 and 100 MHz, respectively, were obtained on a JEOL JNM-AL400 spectrometer with internal tetramethylsilane (TMS) as a standard. TLC was performed on precoated silica gel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany), and spots were visualized under UV light at 254 nm. HPLC was performed utilizing a Waters 2695 HPLC (Nihon Waters, Tokyo, Japan) fitted with an Inertsil ODS-3 column (4.6 × 150 mm, 5 μm; GL Sciences Inc., Tokyo, Japan), and compound was detected at 254 nm. Riboflavin and abietic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Neoabietic acid was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Methyl abietate and pimaric acid were purchased from Chem Service, Inc. (West Chester, PA, U.S.A.) and MP Biomedicals, Inc. (Irvine, CA, U.S.A.), respectively.

Medicinal Plant Material —— The medicinal plant samples of Resina Pini of *Pinus* sp. and each of medicinal plant species, *Chelidonium majus* L. (*C. majus*, Papaveraceae), *Equisetum hyemale* L. (*E. hyemale*, Equisetaceae), *Triticum aestivum* L. (*T. aestivum*, Poaceae), *Polygonum aviculare* L. (*P. aviculare*, Polygonaceae), *Polygonum cuspidatum* Sieb. et Zucc. (*P. cuspidatum*, Polygonaceae), *Polygonum multiflorum* Thunb. (*P. multiflorum*, Polygonaceae), and *Pulsatillae cernua* (*P. cernua*, Ranunculaceae), were purchased from the Herbal Cosmeceutical Material Bank, Korea National Research Resource Center. Voucher specimens have been deposited at the herbarium of the Natural Products Chemistry Laboratory, Department of Herbal Pharmaceutical Engineering, College of Herbal Bioindustry, Daegu Haany University, Republic of Korea.

Preparation of Extracts and Isolation of Active Compounds —— The dried Resina Pini of *Pinus* sp. (500 g) was extracted with 50% ethanol (2 l × 2) at room temperature for 3 days. After filtration, the extract was evaporated under reduced pressure and lyophilized to give a 50% ethanol extract (29.2 g). The other medicinal plant species, *C. majus*, *E. hyemale*, *T. aestivum*, *P. aviculare*, *P. cuspidatum*, *P. multiflorum*, and *P. cernua*, were extracted in a similar manner to afford 50% ethanol extracts, which were used for assay of inhibition against testosterone 5α-reductase. The 50% ethanol extract was partitioned between ethyl acetate and water to give an ethyl acetate-soluble fraction and an aqueous fraction, respectively. The ethyl acetate-soluble fraction was then separated by repeated preparative TLC with the guidance of rat prostate testosterone 5α-reductase inhibitory activity. The active fraction was further purified by recrystallization from ethanol to give the active component, compound **1**, as a yellow powder (1.33 g). The powder was determined to contain exclusively compound **1** using an HPLC system with an octadecylsilane (ODS) column and methanol/water/trifluoroacetic acid (80 : 20 : 0.1).

Enzymatic Assay —— A homogenate of the ventral prostate gland of male Sprague-Dawley rats was prepared, and testosterone 5α-reductase inhibition was measured using the methods previously reported.[5] Both the type 1 and the type 2 isozymes are present in the ventral prostate of the rat. In the assay, since rat prostate extracts were used in a neutral pH buffer, both isozymes were assayed. The ventral prostate glands were excised and minced with a pair of scissors. The 20 w/v% homogenates were prepared with a glass-glass homogenizer in medium A (sucrose 0.32 M, dithiothreitol 0.1 mM, and sodium phosphate 20 mM, pH 6.5). The homogenate was filtered with surgical gauze and then centrifuged at 3000 × g for 10 min. The pellets were suspended in medium A at a protein concentration of 20 mg/ml by triturating sequentially through an 18-gauge and then a 20-gauge needle. To 40 μl of the enzyme suspension, 10 μl of test sample in ethanol or ethanol alone and a mixture containing 0.525 ml of reaction solution consisting of dithiothreitol 1 mM, sodium phosphate 40 mM, pH 6.5, NADPH 5×10^{-5} M, and [1,2,6,7-^{3}H]testosterone 2.2×10^{-9} M were added. The assay mixture was incubated at 37°C for 30 min. The reaction was stopped by the addition of 1 ml of ethyl acetate, and 50 μl of the upper phase was separated on a silica gel plate in the developing solvent system of ethyl acetate/cyclohexane, 1 : 1. The zones corresponding to the testosterone and the 5α-dihydrotestosterone were cut into fragments. Each fragment was added to 5 ml of Aquasol-2 and counted with a liquid scintillation counter to determine ^{3}H radioactivity.

The inhibitory activity of each test sample was calculated as follows:

$$\text{Rate of enzyme reaction } (R, \%) = D/(T + D) \times 100$$

$$\text{Inhibitory activity } (\%) = (1 - R_{\text{sample}}/R_{\text{control}}) \times 100$$

Where D and T are the ^{3}H radioactivity recovered

in the areas of 5α-dihydrotestosterone and testosterone, respectively, in which a substrate blank (substrate minus enzyme) correction is subtracted in each enzyme assay. R_{sample} and $R_{control}$ are R in the presence and absence of test sample, respectively. IC_{50} was calculated from the inhibitory activity values at several concentrations using linear regression analysis. Each enzyme reaction was carried out in duplicate.

Abietic Acid —— Partly crystalline yellow plates; UV λ_{max} (MeOH) nm (ε): 241.0 (22000), 242.2 (22000); ^{1}H-NMR δ (400 MHz, $CDCl_3$): 0.83 (s, 3H, 20-Me), 1.00 (d, J = 4.0 Hz, 3H, 16-H), 1.02 (d, J = 3.2 Hz, 3H, 17-H), 1.27 (s, 3H, 19-Me), 5.39 (s, 1H, 7-H), 5.77 (s, 1H, 14-H), 9.30 (s, 1H, COOH); ^{13}C-NMR δ (100 MHz, $CDCl_3$): 14.0 (C-20), 16.8 (C-19), 18.0 (C-2), 20.9 (C-16), 21.4 (C-17), 22.5 (C-11), 25.6 (C-6), 27.4 (C-12), 34.5 (C-10), 34.9 (C-15), 37.2 (C-3), 38.3 (C-1), 44.9 (C-5), 46.3 (C-4), 50.9 (C-9), 120.5 (C-7), 122.4 (C-14), 135.8 (C-8), 145.1 (C-13), 185.4 (C-18); MS *m/z* [relative intensity (rel. int.) %]: 316 (52), 301 (M^+, 70), 285 (87), 253 (60), 239 (68), 237 (77), 195 (57), 167 (52), 149 (100), 123 (54), 121 (89), 109 (57), 105 (54), 91 (62), 55 (64).

RESULTS AND DISCUSSION

In the course of the search for medicinal plant sources used in several remedies against androgen-dependent diseases including benign prostatic hyperplasia which have testosterone 5α-reductase inhibitory activity, the 50% ethanol extract of Resina Pini of *Pinus* sp. showed potent testosterone 5α-reductase inhibitory activity (Table 1). A 500 μg/ml solution of the dried 50% ethanol extract of Resina Pini of *Pinus* sp. showed a 78.2% inhibition of the enzyme. The inhibitory activity of the 50% ethanol extract of this crude drug was superior to that of each of several medicinal plant species, *C. majus*, *E. hyemale*, *T. aestivum*, *P. aviculare*, *P. cuspidatum*, *P. multiflorum*, and *P. cernua*, all of which have been used in phytotherapeutic preparations for the treatment of benign prostatic hyperplasia. When the 50% ethanol extract was partitioned between ethyl acetate and water, the ethyl acetate-soluble portion exhibited inhibition of the enzyme. Further, when the 50% ethanol extract was adjusted to pH 3.0 and pH 9.0 by respective treatment with acid and alkali and then extracted with ethyl acetate, the portion responsible for the inhibition of the enzyme was extractable exclusively with acid treatment. The ethyl acetate-extractable portion was concentrated by repeated extraction with aqueous alkaline solution and an acidified organic phase. The portion was separated by repeated preparative TLC with the guidance of rat prostate testosterone 5α-reductase inhibitory activity, followed by recrystallization and HPLC to give the active component, compound **1**. Compound **1** in methanol showed characteristic UV absorption peaks at 241.0 and 242.2 nm (ε 22000, 22000), indicating the existence of the CH_2=CHCH=CH_2 moiety within the compound. The electron ionization (EI)-MS of the compound showed characteristic fragmentation at *m/z* 301 (M^+, 70%), which can be assigned to the molecular ion corresponding to abietic acid. Compound **1** was finally identified by inspection of ^{1}H- and ^{13}C-NMR spectra as a diterpene resin acid, abietic acid (Fig. 1).[11]

Table 1. Inhibitory Effects of 50% Ethanol Extracts from Several Crude Drugs of Medicinal Plant Species on Testosterone 5α-Reductase from the Rat Ventral Prostate Gland

Medicinal plant	Dose (μg/ml)	Inhibition (%)
Resina Pini of *Pinus* sp. (Pinaceae)	50	0.8
	500	78.2
C. majus (Papaveraceae)	500	3.4
E. hyemale (Equisetaceae)	500	41.6
T. aestivum (Poaceae)	500	23.5
P. aviculare (Polygonaceae)	500	45.3
P. cuspidatum (Polygonaceae)	500	44.4
P. multiflorum (Polygonaceae)	500	52.6
P. cernua (Ranunculaceae)	500	31.0

Rosin (colophony), Resina Pini of *Pinus* sp., is the residue left after distilling off the volatile oil from the oleoresin (turpentine) obtained from *Pinus* sp., *Pinus palustris* Miller, *Pinus pinaster* Aiton, *Pinus sylvestris* Linne, *Pinus laricio* Poiret, *Pinus longifolia* Roxburgh, *Pinus densiflora* Siebold et Zuccarini, and *Pinus thunbergii* Parlatore. About 90% of the rosin is resin acids, and about 90% of resin acids is abietic acid. Abietic acid is used as an ingredient in pharmaceutic aids and in the manufacture of soaps, waxes, printing inks, paper sizings, *etc*. Abietic acid is also used to assist the growth of lactic and butyric acid bacteria. The isolated abietic acid was evaluated for the inhibitory activity against rat prostate testosterone 5α-reductase. Abietic acid exhibited inhibitory activity in a dose-dependent manner, and showed inhibition of 3.9%,

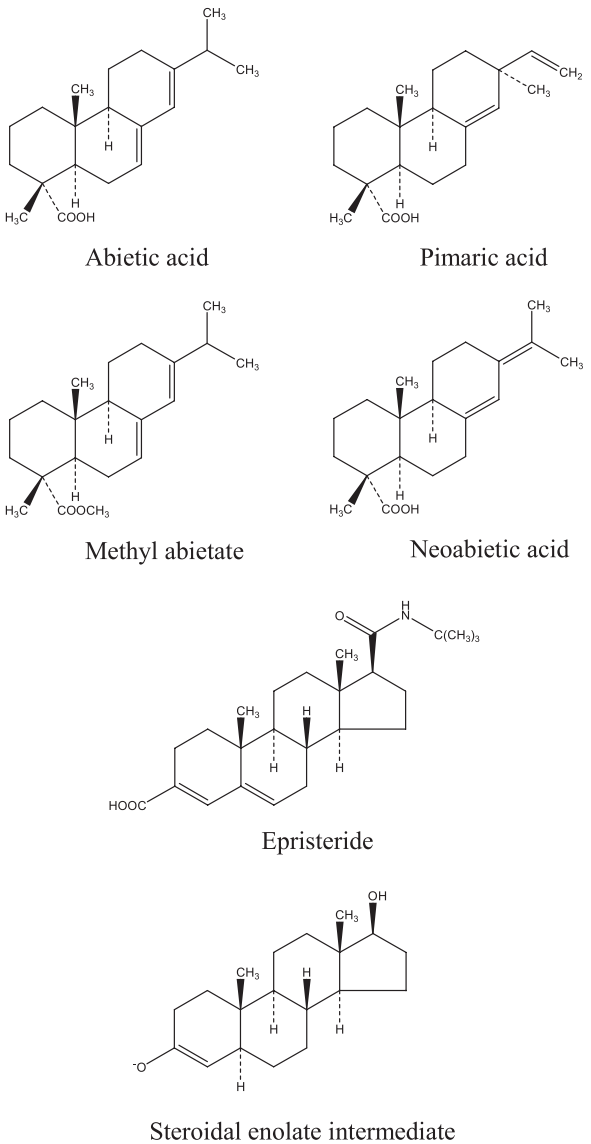

Fig. 1. Chemical Structures of Abietic Acid from the Resina Pini of *Pinus* sp., Several Analogues of Abietic Acid, Methyl Abietate, Pimaric Acid and Neoabietic Acid, Episteride, and Steroidal Enolate Intermediate

22.6%, 59.3%, and 95.9% at concentrations of 1, 10, 100, and 1000 μM, respectively (Table 2). Although abietic acid showed much less potent inhibitory activity than finasteride, the inhibitory activity of the compound was on the same order as that of riboflavin, in which finasteride and riboflavin were used as a steroidal and a nonsteroidal positive control, respectively.[1,12] Methyl abietate, which is the methyl ester of abietic acid, was substantially inactive against testosterone 5α-reductase, indicating that the carboxyl group in the structure of abietic acid is an important structural moiety for the inhibitory activity. Consistent with this concept,

Table 2. IC_{50} Values of Abietic Acid from the Resina Pini of *Pinus* sp. Several Analogues of Abietic Acid, Methyl Abietate, Pimaric Acid and Neoabietic Acid, Riboflavin, and Finasteride on Rat Prostate Testosterone 5α-Reductase Activity

Compound	IC_{50} (μM)
Abietic acid	56 ± 22
Methyl abietate	> 1000
Pimaric acid	750 ± 120
Neoabietic acid	120 ± 50
Riboflavin	15 ± 13
Finasteride	0.06

Each value represents the mean ± S.E. of four experiments.

other diterpene resin acids like pimaric acid and neoabietic acid also showed significant inhibitory activity against testosterone 5α-reductase, indicating that the negatively charged anionic carboxyl group on the molecule is the structural determinant for the inhibitory activity. On the basis of the theory of enzyme catalysis, high-energy transition states or intermediates bind more tightly to enzymes than ground-state substrates or products in enzymatic reaction.[13] The proposed mechanism for testosterone 5α-reductase postulates an enolate as a high energy intermediate of the steroid component.[14] During the testosterone 5α-reductase reaction, these anionic resin acid-type inhibitors like abietic acid may represent greatly simplified transition state analogues similar to the enolate intermediate. Steroidal acrylates such as episteride were designed to mimic the geometry and electrostatic properties of the proposed enolate intermediate.[14] The anionic resin acid-type inhibitors such as abietic acid possess a one-carbon extension in the form of a carboxylic acid at position 4 rather than position 3, suggesting that this element of these compounds approximates more regiospecifically than the steroidal acrylates such as episteride to the oxyanion at position 3 of the steroidal enolate intermediate (Fig. 1).[15] Thus it may be necessary to examine the activity of a series of analogues of these anionic diterpene compounds to study the structure-activity relationship for enhancing inhibitory activity. These findings suggest that a nonsteroidal anionic diterpene compound of natural origin may have the potential to act as a transition state analogue inhibitor of testosterone 5α-reductase in the treatment of androgen-dependent diseases.

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