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Structure–activity relationship for inhibition of 5α-reductase by triterpenoids isolated from *Ganoderma lucidum*

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Abstract—In humans, 5α-reductase is involved in the development of benign prostatic hyperplasia. Triterpenoids isolated from ethanol extracts of *Ganoderma lucidum* (Fr.) Krast (Ganodermataceae) inhibited 5α-reductase activity. The presence of the C-3 carbonyl group and of the C-26-α,β-unsaturated carbonyl group was characteristic of almost all inhibitors isolated from *G. lucidum*.

1. Introduction

The microsomal enzyme steroid 5α-reductase catalyzes the NADPH-dependent reduction of the $\Delta^{4,5}$ double bonds of a variety of 3-oxo-Δ^4 steroids.[1] It is well documented that androgen-responsive tissues such as prostate, seminal vesicle, epididymis, and skin metabolize the conversion of testosterone to 5α-dihydrotestosterone (DHT).[2,3] This process amplifies the androgenic response, perhaps because of the higher affinity of the androgen receptor for DHT than for testosterone.[4] Both 5α-reductase and DHT perform critical roles physiologically and pathologically in humans. For example, DHT is necessary for adult prostate enlargement,[5] for the development of the male genitalia, and for normal beard growth,[6] while administration of DHT can enlarge the undetectable prostate[7] in males born with a genetic 5α-reductase deficiency.[8] Elevated DHT plasma levels have been reported in patients with either benign prostatic hyperplasia (BPH) or prostatic cancer.[9] Therefore, inhibition of androgen action by 5α-reductase inhibitors is a logical treatment for 5α-reductase activity disorders. Furthermore, with the assistance of modern methods of molecular biology, two types of 5α-reductases, identified as types 1[10] and 2,[11,12] have been isolated from human and rat prostatic cDNA libraries, and the structures of both genes have been elucidated. The type 1 isozyme has a broad basic pH optimum and low affinity for testosterone ($K_m > 1$ μM), while the type 2 isozyme has an acidic pH optimum and high affinity for testosterone ($K_m < 10$ nM).[13] The average sequence identity between isozymes within a given species is about 47%, while the sequence identity between the same isozyme across species is 60% for 5α-reductase type 1 and 77% for 5α-reductase type 2.[14] Early reports found that the type 1 isozyme predominates in tissues such as liver, kidney, brain, lung, and skin, whereas the type 2 isozyme is more abundant in genital tissues such as the prostate. However, some recent evidence shows that, in the human prostate, type 1 is expressed mainly in the epithelial cells, whereas type 2 is localized mainly in the stromal compartment.[13,15] Consequently in advanced prostate cancer, which is characterized by the abnormal proliferation of epithelial cells, type 1 might become the predominant isozyme probably responsible for androgen metabolism. Moreover, it has been shown that 5α-reductase type 1 activity is three to four times greater in malignant hyperplasia than in BPH, but 5α-reductase type 2 activity is similar in both diseases. Therefore, we focused on 5α-reductase type 1 activity.

The inhibition of 5α-reductase with organic molecules has been studied for more than two decades. Numerous nonsteroidal and steroidal compounds have been designed and synthesized as competitive, noncompetitive or uncompetitive inhibitors of 5α-reductase. Among them, benzonolinones[16,17] and 4-azasteroids[18,19] have high inhibitory potencies to type 1 and/or type 2 enzyme(s) in vitro and/or in vivo. Finasteride, a synthetic 5α-reductase inhibitor, is currently used to treat

Keywords: 5α-Reductase; *Ganoderma lucidum*; Anti-androgen activities; Benign prostatic hyperplasia (BPH).
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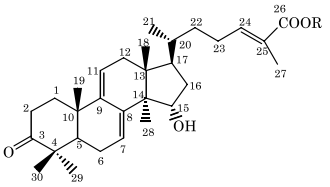
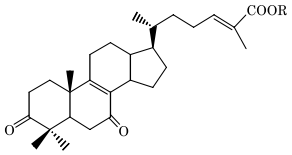
BPH.[20] However, it should be noted that these inhibitors have the potential to cause adverse effects such as those reported for finasteride[21]—that is, gynecomastia, impairment of muscle growth and severe myopathy—due to the structural similarity to steroidal hormones. Hence, the emergence of therapeutic materials having fewer side effects—preferably, edible natural products—would be highly desirable if their safety could be guaranteed.

For thousand of years, mushrooms have been known as a source of medicine. In East Asia, the fruiting body of the fungus *Ganoderma lucidum* has been used for centuries as a folk medicine to treat various human diseases such as cancer, hypertension, hepatitis, nephritis, and so on.[22] Although the proliferation and migration of prostate cancer cells[23] have been reported, the 5α-reductase inhibitory activity of these compounds from *G. lucidum* was reported only by our group. In our last paper, we revealed a characteristic fraction containing triterpenoids after separation by silica gel column chromatography that showed significant 5α-reductase inhibitory activity. We also reported the isolation of oxygenated lanostane-type triterpenoids with 5α-reductase inhibition, ganoderic acid TR, ganoderic acid DM, and 5α-lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one from *G. lucidum*, as well as their inhibitory effects on 5α-reductase.[24,25] In this paper, we investigated the ability



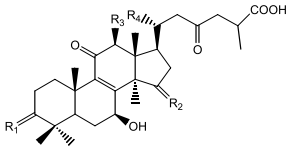
ganoderic acid TR (**1**)

methyl ester of **1** (**14**)

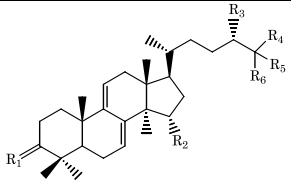


ganoderic acid DM (**2**)

methyl ester of **2** (**15**)



constituent	R_1	R_2	R_3	R_4
ganoderic acid A (**3**)	O		H	H
ganoderic acid B (**4**)		O	H	H
ganoderic acid C2 (**5**)			H	H
ganoderic acid D (**6**)	O	O	H	H
ganoderic acid I (**7**)		O	H	OH



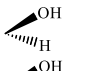
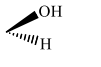
constituent	R_1	R_2	R_3	R_4	R_5	R_6
5α-lanosta- 7,9(11),24- triene-15α,26- dihydroxy-3-one (**8**)	O	OH	$\Delta^{24(25)}$		CH_2OH	CH_3
ganoderol B (**9**)		H	$\Delta^{24(25)}$		CH_2OH	CH_3
ganodermatriol (**10**)		H	$\Delta^{24(25)}$		CH_2OH	CH_2OH
ganodermanontriol (**11**)	O	H	OH	OH	CH_2OH	CH_3
ganoderiol A (**12**)		H	OH	OH	CH_2OH	CH_3
ganoderiol F (**13**)	O	H	$\Delta^{24(25)}$		CH_2OH	CH_2OH

Figure 1. The structure of **1**–**13**.

of these compounds to inhibit 5α-reductase to determine what structural elements are important for the potent inhibition of 5α-reductase by this class of compounds in rat liver microsomes.

2. Chemistry

A variety of natural and synthetic compounds have been found to inhibit 5α-reductase. Here we isolated 13 species of triterpenoids from ethanol extracts of *G. lucidem*, and their structures are shown in Figure 1. The ^{13}C NMR data for ganoderic acid TR (**1**), ganoderic acid DM (**2**),[26] ganoderic acid A (**3**),[27] ganoderic acid B (**4**),[28] ganoderic acid C_2 (**5**),[29] ganoderic acid D (**6**),[30] ganoderic acid I (**7**),[29] 5α-lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one (**8**),[31] ganoderol B (**9**),[32] ganodermatriol (**10**),[33] ganodermanontriol (**11**),[33] ganoderiol A (**12**),[33] and ganoderiol F (**13**)[34] were matched with published data.

3. Results and discussion

Since the publication of papers describing the phenotypic characteristics of humans deficient in DHT,[35] ample evidence has accumulated supporting the proposal that this product of testosterone metabolism is the principal androgen for trophic growth and for support of the prostate[36] and the function of the sebaceous gland.[37] In addition, unusually high levels of DHT have been correlated with diseases such as BPH,[38] acne, male pattern baldness,[39] and female hirsutism.[40] Furthermore, recent results with pharmacological models have provided support for the hypothesis that DHT is a potent, tissue-specific androgen.[41–43] From this proposal, the concept of androgen action has evolved into the theory that administration of specific antagonists of DHT could be an effective therapy for these metabolic disorders. One approach toward this goal would be the blockade of DHT biosynthesis. Here, the most attractive target is 5α-reductase, the NADPH-dependent enzyme that converts testosterone into DHT.

Table 1 shows the ability of each of these compounds to inhibit 5α-reductase activity. The IC_{50} of **3**, **4**, **5**, **6**, **7**, **9**, **10**, **11**, **12**, and **13** were not tested because of their poor solubility. Compounds **1**, **2**, and **8** were better inhibitors of 5α-reductase than the other 10 kinds of triterpenoids. It should be noted that α-linolenic acid, a natural compound with 5α-reductase inhibitory activity that was used as a positive control, showed an IC_{50} of 116 μM (32 μg/mL) in our assay system. Table 1 shows that better inhibitors of type 1 5α-reductase were ganoderic acid TR (**1**), ganoderic acid DM (**2**), and 5α-lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one (**8**). The 5α-reductase inhibitory activity of each compound according to concentration is shown in Figure 2. As the concentrations of **1**, **2**, and **8** increased, the residual enzyme activity rapidly decreased but was not completely suppressed. The inhibitory concentration leading to 50% activity loss (IC_{50}) was estimated to be 8.6 μM, 10.6 μM, and 41.9 μM, respectively. Compound **1** showed the strongest inhibitory activity among all the compounds. Although the structure of **8** is similar to that of **1**, the IC_{50} of **1** was five times higher than that of **8**. The only difference in these two compounds is in C-26: **1** has a 26-carboxy and **8** has a 26-hydroxy. These results suggested that a carboxyl group of the 17β-side chain of **1** is essential to elicit the inhibitory activity. The same tendency was also observed between the methyl ester of **1** and **14**,[44] **2** and **15**. In contrast to **1** and **2**, its methyl ester (**14** and **15**) showed much less inhibitory

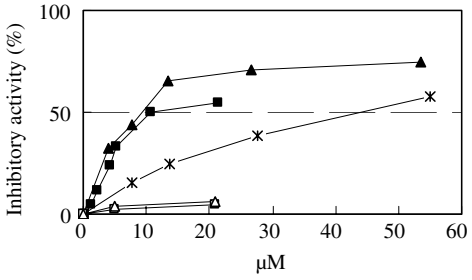


Figure 2. The 5α-reductase inhibitory activity by various concentrations of **1**, **2**, and **8**. (**1**) ▲; (**2**) ■; (**8**) ∗; (**14**) △; (**15**) □.

Table 1. Comparison of the abilities of triterpenes to inhibit 5α-reductase activity (%)

Compound	IC_{50} (μM)	5α-Reductase inhibitory activity (%)		
		667 μM	333 μM	167 μM
Ganoderic acid TR (**1**)	8.6	92		
Ganoderic acid DM (**2**)	10.6	72		
Ganoderic acid A (**3**)		22		
Ganoderic acid B (**4**)		10		
Ganoderic acid C_2 (**5**)		1		
Ganoderic acid D (**6**)		23		
Ganoderic acid I (**7**)		2		
5α-Lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one (**8**)	41.9	89		
Ganoderol B (**9**)		39	37	26
Ganodermatriol (**10**)		39	24	12
Ganodermanontriol (**11**)		32	21	17
Ganoderiol A (**12**)		10	6	0
Ganoderiol F (**13**)		34%	23	16
α-Linolenic acid (positive control)	116			

activity on 5α-reductase, as shown in Figure 2. With this assay, the more potent inhibitory compounds were shown to be associated with analogues that are unsaturated at C-24 and C-25. For example, three most potent inhibitors (**1**, **2**, and **8**) contain sp^2 hybridization from C-24 to C-25, while the fully saturated triterpenoids are less potent. In addition, the C-26-hydroxy (**12**) is less potent than the C-26-carboxy (**1**), indicating that the presence of an acidic functionality at the 26-carbon is imperative for potent enzyme inhibition within this series of compounds. Among ganoderic acids, **1** and **2** were the only ones that have $\Delta^{24,25}$ and showed stronger 5α-reductase inhibitory activity. Among ganoderma alcohol, **8**, **9**, **10**, and **13**, which have $\Delta^{24,25}$, showed stronger 5α-reductase inhibitory activity than those having no $\Delta^{24,25}$ (**11**, **12**) in the 17β-side chain.

The pH dependence of 5α-reductase inhibitory activity of **2** is presented in Figure 3. A high pH is required for 5α-reductase inhibition. Within the pH range (6–8) in the 5α-reductase assay, the conversion from testosterone to DHT by the 5α-reductase was at the same level. These data indicate that the inhibition of 5α-reductase by the 26-carboxy triterpenoids depended on the carboxylate anion of the triterpenoid inhibitor. The protonated, electrophilic form of this functionality might coordinate to the carboxylate anion of the triterpenoid inhibitor through an ionic interaction, thereby stabilizing the enzyme-bound complex.

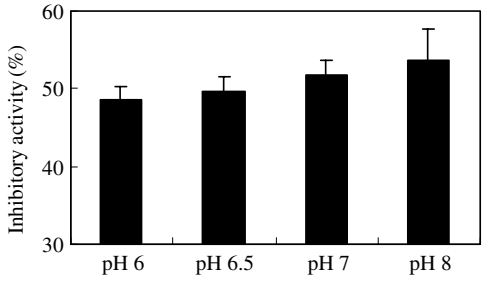


Figure 3. The pH dependence of **2** on 5α-reductase inhibitory activity. $n = 2$.

As a working model, the chemical mechanism of 5α-reductase is proposed to involve the direct hydride transfer from NADPH to C-5 of testosterone, whereby an enzyme-associated electrophile could stabilize the resulting 3,4-enolate (Fig. 4a). The NADPH is the first substance to bind, and the $NADP^+$ is the last product to be released from the enzyme surface. Consequently, both NADPH and $NADP^+$ independently interact with free enzyme, giving rise to binary enzyme complexes to which steroidal inhibitors could associate.[45] Formation of a triterpenoid species with cationic character at C-26 by initial activation of the enone substrate through coordination to this electrophilic center would facilitate the hydride transfer. Upon subsequent C-4 protonation of the enolate intermediate, DHT would be formed. According to Wolfenden,[46] chemically stable structural and electronic mimics of enzyme-bound reaction intermediates should demonstrate high enzyme affinity via exploitation of the specific interactions involved in the stabilization of the active site-associated transition or intermediate states. To optimize the interaction with the binding site of 5α-reductase, the α,β-unsaturated 17β-side chain should therefore be taken into account. Thus, the unsaturated 26-carboxyl triterpenoids should be designed as enzyme-bound compounds (Fig. 4b).

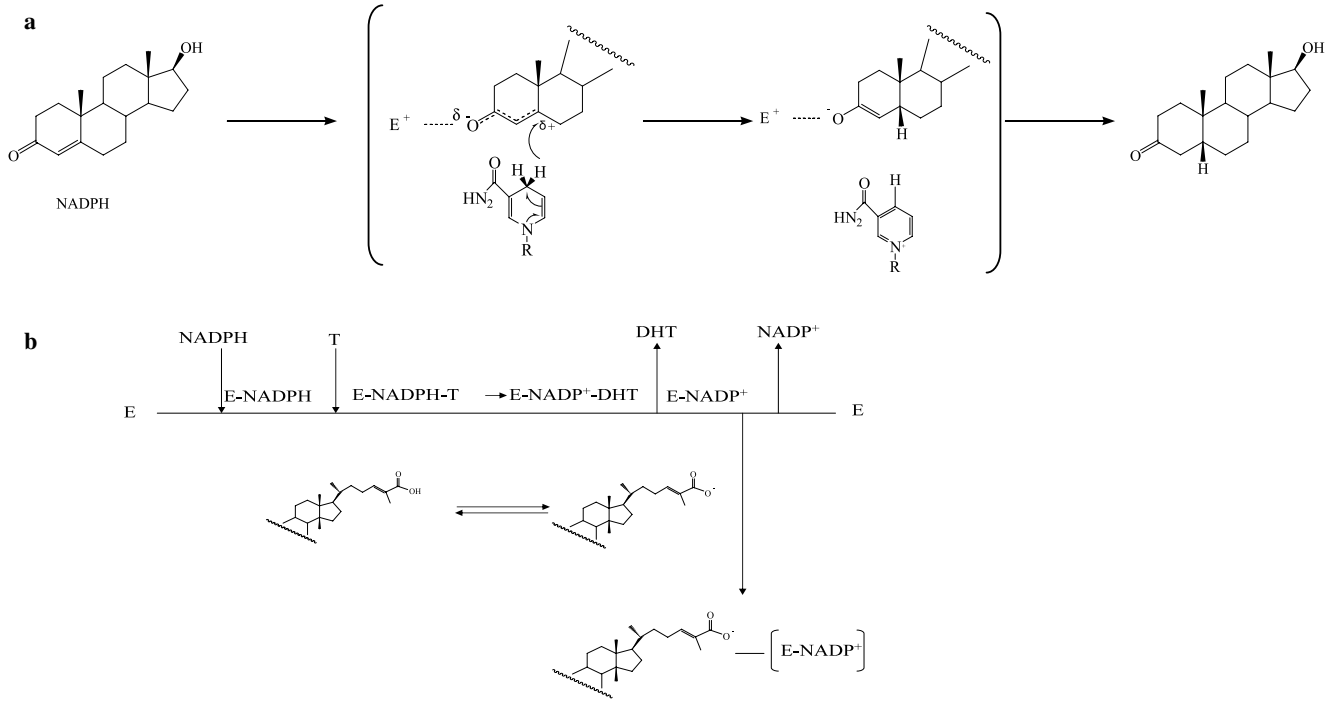


Figure 4. Proposed chemical mechanism of steroid 5α-reductase.

Analysis of various 17β substituents indicated that less-polar functionalities interfered more effectively with the enzyme, but that polarity was not the sole contributing factor in the potency. No ganoderma alcohol was likely to be most effective on these triterpenoid frameworks, but increasing the lipophilicity of the 17β substituents led to maximal inhibitory activity (**9** and **10**). The more hydroxyl the lower inhibitory activity was observed. The hydrophilic compounds did not affect the inhibition of 5α-reductase. Since 5α-reductase is a hydrophobic enzyme, the hydrophobic bond between 5α-reductase and the compounds may make to be tighter. The tendency for the C-3 carbonyl group compound to have a high inhibitory activity was seen between **11** and **12** as well as between **9** and **12**. The mechanism of inhibition by these 3-carbonyl A-ring triterpenoids confirms that C-3 anionic functionality is a primary factor in targeting an inhibitor toward the steroid 5α-reductase-$NADP^+$ complex and in determining inhibitor potency.

4. Conclusion

Factors independent from those related to enzyme-bound intermediates, such as the C-17 substituent, can greatly influence ligand affinity; within a series, however, those compounds that can best mimic the enolate intermediate (Fig. 4) in the testosterone-to-DHT transformation are the better 5α-reductase inhibitors. In this vein, the presence, placement, and degree of unsaturation have been shown to be critical to the potency of 5α-reductase inhibition by 26-carboxy triterpenoids. However, the functionalization that influences binding affinity to 5α-reductase, such as the C-7, C-11 substituent and the lipophilicity of C-17 substituent, has minimal influence on the enzyme form with which the inhibitor associates. It is the C-3 carbonyl, $\Delta^{24,25}$, and C-26 carboxy that serve as primary binding determinants to the enzyme–$NADP^+$ complex, as demonstrated by comparisons between the dead-end and multiple-inhibition results.

In the last few years, the use of herbal therapies in alternative medicine has increased. Although the number of cancer patients using herbal dietary supplements is not exactly known, the evidence of the increasing use of dietary supplements in cancer treatment is reported.[47] *G. lucidum* is part of the herbal mixture PC-SPES, which showed activity against hormone-refractory disease in two prostate cancer patients.[48] Extracts of PC-SPES demonstrated estrogenic effects[47] and decreased growth of hormone-sensitive as well hormone-insensitive prostate cancer cells. In light of our results, these effects might be related not only to anti-cancer effects of *G. lucidum* but also to anti-androgen effects. Since excessive 5α-reductase activity has been proposed to be a possible contributing factor in prostate cancer development or progression,[49] the development and progression of prostate cancer may also be affected by diets containing inhibitors of 5α-reductase.

We performed this structure–activity relationship study of 5α-reductase inhibitors in an effort to provide some natural product inhibitors of this enzyme. Inhibition studies were conducted using a rat live microsomal assay. This assay provides some estimate of the potential of a particular compound to inhibit 5α-reductase activity *in vitro*. This study identified several natural products that were inhibitors of 5α-reductase. *In vivo* experimentation will be needed to confirm the effects of these compounds and to make sure that active levels of these compounds are supplied to target tissues. If some of these compounds are active *in vivo*, they may be important candidates for BPH and prostate cancer therapy.

5. Experimental

5.1. Chemistry

Ganoderma lucidum was obtained from Bisoken (Fukuoka, Japan). The mushroom was identified by Mr. Shuhei Kaneko, Fukuoka Prefecture Forest Research and Extension Center. The fruiting body was dried and ground to powder before use. Unless otherwise specified, chemicals were obtained from Sigma Aldrich Japan (Tokyo, Japan). Organic solvents were purchased from Wako Pure Chemical Industries (Osaka, Japan). [4-^{14}C]Testosterone was obtained from PerkinElmer Japan (Kanagawa, Japan).

5.2. Ethanol extracts of *G. lucidum*

Dried and chipped *G. lucidum* (15 kg) was extracted with 95% ethanol (126 L) at room temperature for 24 h in a blender. The extracts were filtered through ADVANTEC No. 2 filter paper, concentrated under vacuum, and freeze dried. The extracts (571.1 g) were stored in −20 °C before assay.

The 95% EtOH extract (571 g) was fractionated into three fractions [Fr. A (240 g), Fr. B (35 g), and Fr. C (269 g)] (Fr. A: TLC, silica gel, I_2 detection, EtOAc/*n*-hexane, 7:3, R_f 0.48–0.97, Fr. B: R_f 0.03–0.67, and Fr. C: R_f 0–0.04) by column chromatography on silica gel. Repeated column chromatography of Fr. B led to the isolation of four compounds: ganoderic acid TR (**1**), ganoderic acid DM (**2**), 5α-lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one (**8**) and ganoderol B (**9**), as identified by comparing the MS, NMR, and optical rotation matched with published data (Fig. 1).

Dried and chipped *G. lucidum* (200 g) was extracted with 30% EtOH at room temperature for 24 h in a blender. The extracts were filtered through ADVANTEC No. 2 filter paper, concentrated under vacuum, and freeze dried. The extracts (10 g) were stored at −20 °C before assay.

The 30% EtOH extracts (10 g) were suspended in water (0.3 L) and extracted with $CHCl_3$ (5× 1 L), water-saturated BuOH (5× 2 L) successively. Repeated column chromatography of the $CHCl_3$-soluble fraction and BuOH-soluble fraction led to the isolation of nine compounds: ganoderic acid A (**3**), ganoderic acid B (**4**), ganoderic acid C_2, ganoderic acid D (**6**), ganoderic acid I

(**7**), ganodermatriol (**10**), ganodermanontriol (**11**), ganoderiol A (**12**), and ganoderiol F (**13**), as identified by comparing MS, NMR, and optical rotation matched with published data (Fig. 1).

5.3. Preparation of rat microsomal

Rat liver from female SD rat (7 weeks old) were each prepared by a method previously reported by Shimizu et al. with some modifications.[50] Two mature SD female rats were killed. The liver was removed and minced tissue was homogenized in a 4-tissue volume medium A (0.32 M sucrose, 1 mM dithiothreitol, and 20 mM sodium phosphate, pH 6.5). The homogenate was then centrifuged at 10,000*g* for 10 min. The resulting supernatant from the centrifugations was further centrifuged at 105,000*g* for 1 h twice. The washed microsomes were suspended in 1-pellet volume medium A, and the dispersion of microsomes was achieved using a syringe with 18 G, 23 G, and 26 G needles in succession. The microsomal suspension was stored at −70 °C just before use.

5.4. Measurement of 5α-reductase inhibitory activity

A complete reaction mixture included 1 mM dithiothreitol, 20 mM phosphate buffer (pH 6.5), 1.9 nCi [4-^{14}C]testosterone, 150 μM testosterone, 167 μM NADPH, and the enzyme preparation (1.54 mg of protein) in a final volume of 0.3 mL. The concentration of testosterone contributed by [4-^{14}C]testosterone was negligible. Triterpenoid was added at each concentration. The incubation was carried out for 10 min at 37 °C. The incubation was started by the addition of 10 μL microsomes to pre-heated reaction solution in a tube. After 10 min, the incubation was terminated by adding 10 μL of 3 M NaOH. To extract metabolites, 1 mL of diethyl ether was added, and the tubes were capped and shaken. The organic phase was applied to a silica plate (Kieselgel 60 F_{254}). The plate was developed in ethyl acetate/*n*-hexane (7:3) at room temperature. The radioactivity profile was determined with an imaging analyzer (FLA-5000 RF, Fuji Film, Tokyo, Japan). The 5α-reductase activity was calculated from the percentage of the extent to which [4-^{14}C]testosterone was converted to [4-^{14}C] DHT.

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