A Novel Testosterone 5α-Reductase Inhibitor, 8′,9′-Dehydroascochlorin Produced by *Verticillium* sp. FO-2787

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A new testosterone 5α-reductase inhibitor, 8′,9′-dehydroascochlorin, was isolated from the cultured mycelium of *Verticillium* sp. FO-2787. This compound (M.W.: 402, $C_{23}H_{27}ClO_4$) consists of 5-chloroorcylic aldehyde and 2,3,4-trimethylcyclohexenone moieties, which are connected *via* a chain of (2*E*,4*E*)-3-methyl-2,4-pentadiene. The IC_{50} value of the new microbial metabolite for testosterone 5α-reductase activity prepared from rat prostate was 4×10^{-4} M.

Keywords microbial metabolite; testosterone 5α-reductase inhibitor; 8′,9′-dehydroascochlorin; *Verticillium*; cultured mycelium

Androgens, particularly 5α-dihydrotestosterone (DHT), play a key role in the differentiation, growth and maintenance of the mammalian prostate. Benign prostate hyperplasia has been associated with an overproduction of DHT which is converted from testosterone by testosterone 5α-reductase (T-5α-reductase).[1] In the course of our search for new nonsteroidal inhibitors of T-5α-reductase, 8′,9′-dehydroascochlorin was discovered from the cultural mycelium of *Verticillium* sp. FO-2787, together with five known compounds, LL-Z 1272ε (**1**), LL-Z 1272γ (ascochlorin, **2**), LL-Z 1272β (**3**), LL-Z 1272ζ (**4**), LL-Z 1272δ (**6**) (Fig. 1). We report here the isolation of 8′,9′-dehydroascochlorin and substances related to ascochlorin with details of their structural determination and T-5α-reductase inhibitory effects.

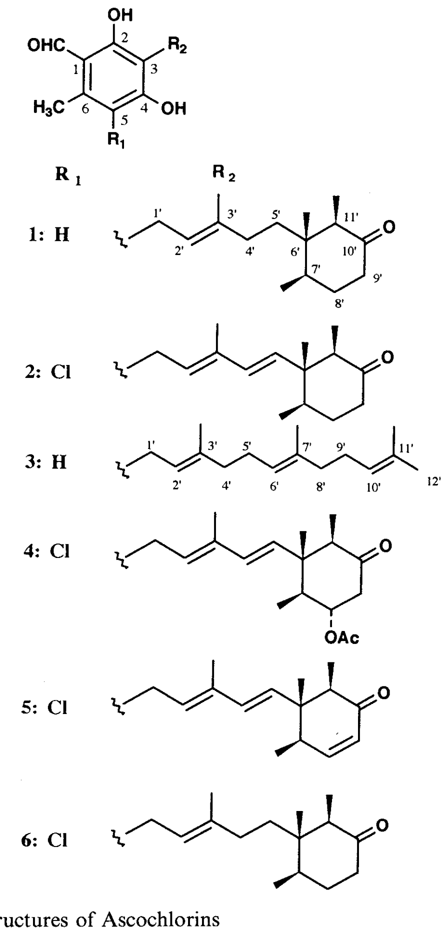

Fig. 1. Structures of Ascochlorins

Strain FO-2787 isolated from a soil sample collected in Shizuoka prefecture, Japan, was determined to be a *Verticillium* species from morphological studies. From the ethyl acetate extracts of cultured mycelium, six metabolites (**1**—**6**) were isolated by repeated silica gel column chromatography and by preparative HPLC with the guidance of T-5α-reductase inhibitory activity. Among these six compounds, five compounds (**1**—**4**, **6**) were reported as antiviral and anti-*Tetrahymena pyriformis* by a number of investigators.[2,3]

A new inhibitor (**5**), white plates, mp 127—130 °C, $[\alpha]_D^{24}$ $-80°$ ($c=0.5$, MeOH), showed characteristic UV absorption of an α,β-unsaturated carbonyl at 348.0 nm (log ε, 3.95) and 292.4 nm (log ε, 3.91). The molecular formula was determined to be $C_{23}H_{27}ClO_4$ (M^+, *m/z* 402.1590, Calcd 402.1598) by the high resolution-electron impact (HR-EI) mass spectrum, which was a corresponding derivative less 2 mass units from ascochlorin (**2**) [$C_{23}H_{29}ClO_4$]. The fragmentation at *m/z* 199 (100%) in the EI mass spectrum of **5** can be assigned to the peak corresponding to the 5-chloroorcylic aldehyde moiety.[4] In the ^{1}H-NMR spectrum of **5**, signals appearing at δ 12.71 (1H, s) and δ 10.15 (1H, s) were assigned to a chelated phenolic proton (C-2) and an aldehyde proton (C-1), respectively, which were in good agreement with those of **2** (Table I).[5] The signal observed at δ 3.54 (2H, d, $J=7.6$ Hz) was assigned to benzylic protons adjacent to an olefinic proton measured at δ 5.55 (1H, t, $J=7.6$ Hz). Furthermore, two doublet-methyl signals at δ 0.95 and 0.98 (3H, d each) were characteristic of the cyclohexanone moiety of ascochlorins.[5] In the ^{1}H–^{1}H correlation spectroscopy (^{1}H–^{1}H COSY) spectrum, the signal of an olefinic proton at δ 5.99 (2H, dd-like, $J=16.2$, 3.3 Hz) was coupled with that of δ 6.56 (1H, dd, $J=9.9$, 2.0 Hz) and that of δ 5.42 (1H, d, $J=16.2$ Hz). The coupling system (1H d, $J=16.2$ Hz, each) at δ 5.99 and 5.42 is a typical AB type doublet assignable to a *trans* conjugated double

Table I. ^{1}H-NMR Chemical Shifts of **1**—**6** in $CDCl_3$

H	1	2	3	4	5	6
1-CHO	10.06 (1H, s)	10.14 (1H, s)	10.06 (1H, s)	10.14 (1H, s)	10.15 (1H, s)	10.14 (1H, s)
2-OH	12.70 (1H, s)	12.71 (1H, s)	12.73 (1H, s)	12.71 (1H, s)	12.71 (1H, s)	12.69 (1H, s)
4-OH	6.92 (1H, br s)	—	6.44 (1H, br s)	—	—	—
5	6.24 (1H, s)	—	6.21 (1H, s)	—	—	—
6-Me	2.48 (3H, s)	2.60 (3H, s)	2.49 (3H, s)	2.61 (3H, s)	2.61 (4H, s)	2.60 (3H, s)
1′	3.37 (2H, d, 6.6)	3.53 (2H, d, 7.6)	3.40 (2H, d, 7.3)	3.54 (2H, d, 7.6)	3.54 (2H, d, 7.6)	3.39 (2H, d, 7.3)
2′	5.28 (1H, t, 6.6)	5.52 (1H, t, 7.6)	5.27 (1H, t, 7.3)	5.54 (1H, t, 7.6)	5.55 (1H, t, 7.6)	5.25 (1H, dd)$^{d)}$
4′a	2.10—1.83 (1H, m)	5.90	2.03 (1H, m)	5.92 (1H, d, 16.0)	5.99 (2H, dd)$^{b)}$	1.98 (1H, m)
4′b	1.63 (1H, m)	—	2.03 (1H, m)	—	—	1.89—1.77 (1H, m)
5′a	1.40 (2H, m)	5.38 (1H, d, 16.0)	2.03 (1H, m)	5.32 (1H, d, 16.0)	5.42 (1H, d, 16.2)	1.38 (1H, m)
5′b	1.40 (2H, m)	—	2.03 (1H, m)	—	—	1.38 (1H, m)
6′	—	—	5.07 (1H, br s)	—	—	—
7′	2.10—1.83 (1H, m)	1.99—1.87 (1H, m)	—	2.01 (1H, m)	2.61 (1H, s)	1.98 (2H, m)
8′a	2.10—1.83 (1H, m)	1.99—1.87	2.03 (1H, m)	4.89 (1H, m)	6.56 (1H, dd)$^{c)}$	1.89—1.77 (1H, m)
8′b	2.10—1.83 (1H, m)	1.63 (1H, ddd)$^{a)}$	2.03 (1H, m)			1.63 (1H, m)
9′a	2.34 (1H, m)	2.39 (3H, m)	2.03 (1H, m)	2.42—2.38 (1H, m)	5.99 (2H, dd)$^{b)}$	2.31 (1H, m)
9′b	—	—	2.03 (1H, m)	—	—	—
10′	—	—	5.07 (1H, br s)	—	—	—
11′	2.56—2.44	2.39 (1H, m)	—	2.42—2.38 (1H, m)	2.46 (1H, q, 6.8)	2.45 (1H, q, 6.6)
12′	—	—	1.67 (1H, s)	—	—	—
3′-Me	1.83 (3H, s)	1.92 (3H, s)	1.81 (3H, s)	1.92 (3H, s)	1.93 (3H, s)	1.81 (3H, s)
6′-Me	0.57 (3H, s)	0.69 (3H, s)	—	0.73 (3H, s)	0.80 (3H, s)	0.61 (3H, s)
7′-Me	0.92 (3H, d, 6.6)	0.83 (3H, d, 6.6)	1.59 (3H, br s)	0.86 (3H, d, 6.6)	0.95 (3H, d, 6.9)	0.90 (3H, d, 6.9)
11′-Me	0.88 (3H, d, 6.9)	0.81 (3H, d, 6.9)	1.59 (3H, br s)	0.86 (3H, d, 6.6)	0.98 (3H, d, 7.3)	0.88 (3H, d, 5.3)
Ac-Me	—	—	—	2.06 (3H, s)	—	—

a) J=13.2, 12.9, 5.6 Hz; *b)* J=16.2, 3.3 Hz, overlapped; *c)* J=9.9, 2.0 Hz; *d)* J=7.3, 6.2 Hz.

Table II. ^{13}C-NMR Chemical Shifts of **1**—**6** in $CDCl_3$

C	1	2	3	4	5	6
1	113.1 (s)	$113.7^{a)}$ (s)	113.2 (s)	$114.6^{a)}$ (s)	$113.7^{b)}$ (s)	$114.3^{a)}$ (s)
2	163.7 (s)	162.2 (s)	$163.6^{b)}$ (s)	162.2 (s)	162.2 (s)	162.2 (s)
3	112.1 (s)	$113.6^{a)}$ (s)	116.6 (s)	$113.6^{a)}$ (s)	$113.6^{b)}$ (s)	$113.6^{a)}$ (s)
4	162.3 (s)	156.2 (s)	$162.6^{b)}$ (s)	156.1 (s)	156.2 (s)	156.3 (s)
5	110.6 (d)	$113.1^{a)}$ (s)	110.8 (d)	$113.1^{a)}$ (s)	$113.2^{b)}$ (s)	$113.2^{a)}$ (s)
6	141.9 (s)	137.8 (s)	142.0 (s)	137.8 (s)	137.8 (s)	137.6 (s)
1′	21.2 (t)	22.2 (t)	21.2 (t)	22.2 (t)	22.2 (t)	22.1 (t)
2′	121.4 (d)	127.5 (d)	120.9 (d)	128.2 (d)	$128.0^{c)}$ (d)	121.0 (d)
3′	138.0 (s)	134.1 (s)	139.5 (s)	134.1 (s)	134.1 (s)	136.6 (s)
4′	32.6 (t)	133.2 (d)	$39.7^{c)}$ (t)	133.8 (d)	134.0 (d)	42.6 (t)
5′	35.6 (t)	135.6 (d)	$26.6^{d)}$ (t)	135.4 (d)	134.2 (d)	35.6 (t)
6′	43.6 (s)	48.4 (s)	$124.3^{e)}$ (d)	47.2 (s)	47.9 (s)	43.5 (s)
7′	36.0 (d)	40.8 (d)	135.6 (s)	45.6 (d)	41.9 (d)	36.1 (d)
8′	30.9 (t)	31.1 (t)	$39.6^{c)}$ (t)	73.7 (d)	152.1 (d)	31.0 (t)
9′	41.5 (t)	41.5 (t)	$26.2^{d)}$ (t)	45.3 (t)	$127.9^{c)}$ (d)	41.6 (t)
10′	215.0 (s)	212.8 (t)	$123.5^{e)}$ (d)	207.6 (s)	201.4 (s)	214.1 (s)
11′	50.5 (d)	53.6 (d)	131.3 (s)	53.8 (d)	51.8 (d)	50.4 (d)
12′	—	—	25.7 (q)	—	—	—
1-CHO	192.9 (d)	193.2 (d)	193.0 (d)	193.2 (d)	193.2 (d)	193.3 (d)
Methyl group						
6	18.0 (q)	14.4 (q)	18.0 (q)	12.7 (q)	14.4 (q)	14.5 (q)
3′	16.4 (q)	12.6 (q)	16.2 (q)	12.6 (q)	12.7 (q)	16.4 (q)
6′	15.3 (q)	10.3 (q)	—	11.6 (q)	10.0 (q)	15.3 (q)
7′	7.5 (q)	16.3 (q)	16.0 (q)	14.5 (q)	15.1 (q)	7.5 (q)
11′	15.0 (q)	8.9 (q)	17.6 (q)	8.9 (q)	9.0 (q)	15.1 (q)
Acetyl group						
Me	—	—	—	21.0 (q)	—	—
C=O	—	—	—	170.1 (q)	—	—

a—e) Assignments may be interchangeable.

bond. The residual coupling systems (1H, d, J=9.9 Hz) at δ 6.56 and 5.99 were assigned to α,β-unsaturated protons (C-8′, C-9′) in the cyclohexenone moiety of the molecule. The catalytic hydrogenation on 5% Pd/C of **5** gave **2**. From these results, **5** was determined to be 8′,9′-dehydroascochlorin. The ^{13}C-NMR chemical shifts of **5** were also assigned by ^{1}H–^{13}C COSY spectrum (Table II). The relative structure of **5** was anticipated by nuclear

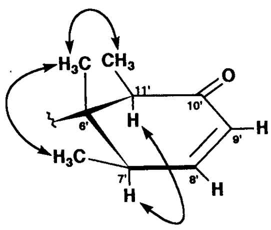

Fig. 2. NOESY Experiments for the Cyclohexenenone Moiety of **5**

TABLE III. CD Cotton Effects of Ascochlorins

Compound	$\Delta\varepsilon$ (nm)	Stereochemistry
1	−1.7 (290)	6′*S*, 7′*R*, 11′*R*
2	−7.3 (290)	6′*R*, 7′*R*, 11′*R*
4	−4.9 (290)	6′*S*, 7′*S*, 8′*S*, 11′*R*
5	−2.2 (327)	6′*R*, 7′*R*, 11′*R*
	−8.3 (244)	
6	−1.3 (290)	6′*S*, 7′*R*, 11′*R*

TABLE IV. Testosterone 5α-Reductase Inhibitory Activities

Compound	IC_{50} (M)
1	3.7×10^{-4}
2	3.4×10^{-4}
3	3.6×10^{-4}
4	3.4×10^{-4}
5	14.0×10^{-4}
6	3.7×10^{-4}
Riboflavin	1.3×10^{-3}

Overhauser effect spectroscopy (NOESY) experiments (Fig. 2). Several correlation peaks between 6′-methyl and 11′-methyl, 6′-methyl and 7′-methyl, and 7′-proton and 11′-proton were observed. Therefore, of the three methyl groups attached to the cyclohexenone ring, two were in equatorial (C-7′, C-11′) and the other in axial position (C-6′). The final confirmation of the absolute configuration of **5** was made by chemical transformation of **5** to ascochlorin (**2**) by catalytic hydrogenation on 5% Pd/C. Ascochlorin (**2**), whose absolute configuration was clarified by X-ray structure analysis,[6,7] showed a negative Cotton effect at 290 nm ($\Delta\varepsilon$, −7.3) on the circular dichroism (CD) spectrum, whereas, **5** showed negative Cotton effects at 327 nm ($\Delta\varepsilon$, −2.2) and at 244 nm ($\Delta\varepsilon$, −8.3). Furthermore, ascochlorin (**2**) derived from **5** gave a negative Cotton effect at 290 nm ($\Delta\varepsilon$, −7.2). These results clearly indicate that **5** has the same absolute configuration series as that of **2** (6′*R*, 7′*R*, 11′*R*). Consequently, the stereochemistry of **5** was confirmed to be 6′*R*, 7′*R*, 11′*R*. Other compounds (**1**, **4**, **6**) also showed similar Cotton effects to that of **2** (Table III). Therefore, a series of cyclohexanone rings of ascochlorin-analogs possesses the same absolute configuration as that of **2**. Compound **5** has already been prepared synthetically by Ellestad *et al.*,[3] but the present study is the first isolation and full characterization of **5** of microbial origin.

The inhibitory effect of compounds (**1**—**6**) on rat prostate testosterone 5α-reductase was tested, and the IC_{50} values of the compounds were 3.4—14.0×10^{-4} M as shown in Table IV. These compounds showed no antimicrobiological activities against gram-positive and gram-negative bacteria, fungi or yeast at a concentration of 500 μg/ml.

As inhibitors of testosterone 5α-reductase activity, several nonsteroidal compounds, including riboflavin, were isolated from the fermentation broth of microorganisms.[8−11] According to our present experiment, the inhibitory activities of **5** and related compounds were more than 10-fold greater than that of riboflavin. The structure of **5** was completely different from that of riboflavin and related compounds. Therefore, it would be of interest to examine **5** in *in vivo* experiments.

Experimental

^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-EX 270 spectrometer (^{1}H 270.05 MHz, ^{13}C 67.8 MHz). The ^{1}H- and ^{13}C-NMR signals were assigned with the aid of the ^{1}H–^{1}H, and ^{1}H–^{13}C COSY, heteronuclear multiple bond connectivity (HMBC) and NOESY experiments. Melting points were measured using a Yanaco micromelting point apparatus and are uncorrected. Kieselgel 60 (70—230 mesh, 230—400 mesh, Merck) was used for column chromatography, and DC-Alufolien Kieselgel 60 F_{254} (Merck) was used for TLC analysis. Analytical and preparative HPLC was carried out with MeOH–H_2O (8 : 2) and CH_3CN–H_2O (8 : 2) using Cosmosil 5C18-AR packed columns (Nacalai Tesque, 5 mm, i.d. 4.6 × 250 mm, i.d. 20 × 250 mm) employing a UV monitoring system (210 or 254 nm). CD spectra were recorded on a JASCO J-720 spectropolarimeter in MeOH.

Testosterone 5α-Reductase Assay The assay mixture consisting of 30 μl of 0.25 M sucrose–0.1 m HEPES buffer (pH 7.4), 10 μl of 50 mM NADPH, 10 μl of 0.75 nmol [^{14}C]testosterone, 30 μl of the test sample, and 20 μl of the enzyme solution prepared from the prostate of Donryu male rats[8] was incubated for 2 h at 37 °C. *n*-Hexane (400 μl) was added, and the mixture was shaken. The *n*-hexane layer (20 μl) was applied to 0.25 mm-precoated silica gel plates (Merck), and the plates were developed with a solvent system of cyclohexane–ethyl acetate (1 : 1). The radioactive areas of [^{14}C]testosterone and [^{14}C]-5α-dihydrotestosterone were determined using an AMBIS image analyzer. Riboflavin was purchased from Kanto Chemical Co., Inc.

Fermentation of Strain FO-2787 and Isolation of Active Compounds A stock culture of the producing organism was inoculated into a test tube (i.d. 2 × 20 cm) containing 10 ml of seed medium consisting of 2% glucose, 0.2% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% polypeptone, 0.1% KH_2PO_4 and 0.1% agar (pH 6.0 before sterilization). The tube was incubated at 27 °C for 72 h on a reciprocal shaker. Then, 2 ml portions of the growth were transferred to a 500 ml Erlenmeyer flask containing 100 ml of the seed medium. The flask was incubated at 27 °C for 72 h on a rotary shaker (210 rpm), and 600 ml of the resulting culture was transferred into a 50-liter fermentor containing 30 l of the production medium consisting of 3% soluble starch, 1% glycerol, 2% soybean meal, 0.3% yeast extract, 0.3% KCl, 0.2% $CaCO_3$, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.05% KH_2PO_4 (pH 6.5). The fermentation was carried out at 27 °C for 96 h using an agitation rate of 160 rpm and an aeration rate of 60 l per minute.

The ethyl acetate extracts of cultured mycelium were chromatographed on a silica gel (70—230 mesh) column using $CHCl_3$–MeOH (9 : 1). Furthermore, each active fractions was submitted to a silica gel (230—400 mesh) column using $CHCl_3$–MeOH (20 : 1→9 : 1) as the developing solvent. Finally, isolation of each compound (**1**—**6**) by use of preparative HPLC gave **1** (80 mg), **2** (60 mg), **3** (30 mg), **4** (6.5 mg), **5** (8 mg) and **6** (8 mg), respectively.

Catalytic Hydrogenation of 5 Compound **5** (1.0 mg) was dissolved in 3 ml of 0.01 N KOH–MeOH and hydrogenated at 1 atm over 5% palladium carbon at room temperature for 5 min. The resulting solution was neutralized by 0.01 N HCl and filtered through a Cosmonice filter (W-type, 0.45 μm, Nacalai Tesque). The product was purified by preparative HPLC (MeOH–H_2O (8 : 2)) to give 0.65 mg of pure **2** (yield, 64.7%). The reaction product was identified as ascochlorin (**2**) by EI-MS, ^{1}H-NMR and co-HPLC with the authentic sample.

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