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Sterenin A, B, C and D, Novel 11β-Hydroxysteroid Dehydrogenase Type 1 Inhibitors from *Stereum* sp. SANK 21205

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Abstract The novel 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) inhibitors known as sterenin A, B, C and D were found in a solid-state culture of the producing basidiomycetes identified as *Stereum* sp. SANK 21205. Purification of the 50% aq Me_2CO extract of the culture was performed by EtOAc extraction, reversed phase open-column chromatography and successive ODS HPLC preparation. These compounds, whose structures were determined by several spectroscopic methods, were found to be novel isoindolinone alkaloids which exhibited potent selective inhibitory activities against 11β-HSD1.

Keywords sterenin, 11β-HSD1 inhibitor, *Stereum*, taxonomy, fermentation, isolation, structure

Introduction

Metabolic syndrome is a general term referring to the accumulation of associated disorders characterized by insulin resistance, obesity, dyslipidemia and hypertension, which are major risk factors for cardiovascular diseases and atherosclerosis. Among the many factors that play a role in this syndrome, glucocorticoids have received considerable attention. Excess glucocorticoid levels cause glucose intolerance and insulin resistance, clinically indicating Cushing's syndrome [1, 2], which closely resembles metabolic syndrome. Accumulated evidence suggests that 11β-hydroxysteroid dehydrogenase (11β-HSD), an enzyme responsible for producing glucocorticoids in tissues, is up-regulated in obesity subjects, including humans [3～5]. Targeted disruption of 11β-HSD in mice exhibits a protective glycemic, lipid and lipoprotein profile. As glucocorticoids oppose the insulin effect, several studies with mice lacking [6] or overexpressing [7] 11β-HSD1 reported that 11β-HSD1 plays an important role in Type 2 diabetes and metabolic syndrome. Thus, an 11β-HSD1 inhibitor is expected to be a promising target in the treatment of metabolic syndrome.

In the course of screening natural products with 11β-HSD1 inhibitory activity, one basidiomycete was found to be the active compound producer designated *Stereum* sp. SANK 21205. Here, we describe the taxonomy of the producing organism, and the fermentation, isolation, structural elucidation and biological activities of sterenin A, B, C and D (Fig. 1), which are novel 11β-HSD1 inhibitors.

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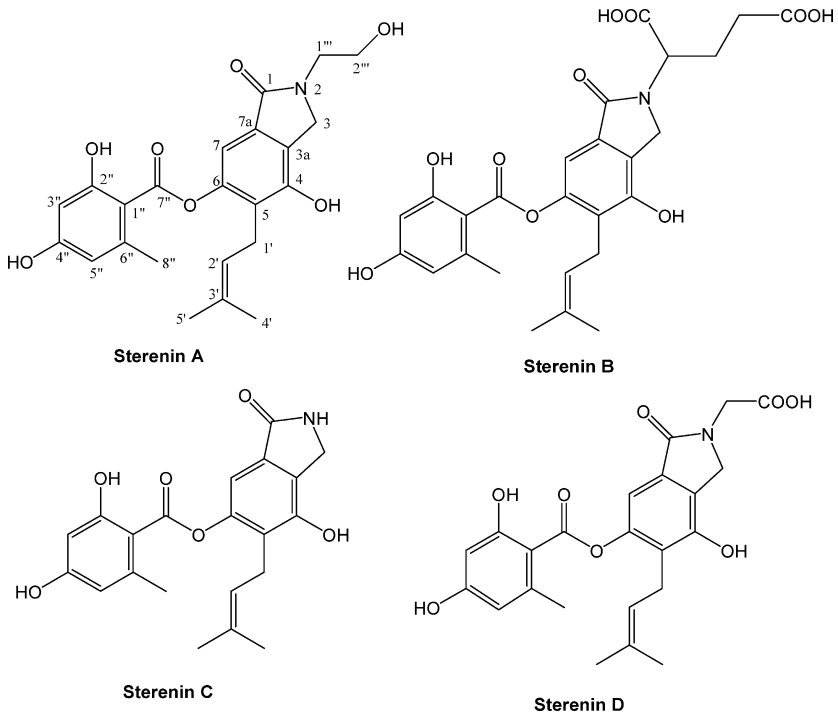

Fig. 1 Structures of sterenin A, B, C and D.

Materials and Methods

General

The ^{1}H- and ^{13}C-NMR spectra were recorded at 298K on an AVANCE 500 spectrometer equipped with a cryogenic probe (Bruker BioSpin) operating at 500 MHz and 125 MHz, respectively. The samples for NMR characterization were dissolved in CD_3OD. The optical rotation and IR spectra were measured with a DIP-370 (JASCO) and a VALOR-III (JASCO), respectively. The mass spectra and high-resolution mass spectra were measured with a JMS700QV Mstation (JEOL). HPLC analysis was carried out with an HP1100 system (Agilent).

Preparation of 11β-HSD1-expressing Microsomes

Human and mouse 11β-HSD1 cDNA were amplified by RT-PCR and sub-cloned into a pCIneo (Promega) expression vector. Expression plasmid was transfected into Hek293 cells using Lipofectamine plus reagent (Invitrogen). At 48 hours post-transfection, the cells were harvested, suspended in Hepes buffer (20 mM Hepes (pH 7.4), 1.0 mM EDTA, 2.0 mM $MgCl_2$ and protease inhibitor cocktail (Roche)) and disrupted by N_2 cavitation at 4.1 MPa. The cell homogenates were centrifuged for 10 minutes at 1,000 g and the pellets were discarded. The supernatants were re-centrifuged at 105,000 g for 30 minutes. The pellets were re-suspended in reaction buffer (50 mM Tris (pH7.4), 10% glycerol).

11β-HSD1 Reductase Assay

The microsomes were incubated in reaction buffer for 3 hours at room temperature (26°C) with an NADPH generation system (0.8 mM NADPH, 6.0 mM glucose-6-phosphate, 0.35 U/ml glucose-6-phosphate dehydrogenase, 3.0 mM $MgCl_2$), respective concentrations of sterenins and 160 nM of substrate (human: cortisone, mouse: 11-dehydrocorticosterone). The reaction was stopped by adding 50 μM of carbenoxolone and the product (human: cortisol, mouse: corticosterone) concentration was determined using HTRF Cortisol Assay Reagents (Cisbio).

Preparation of 11β-HSD2-expressing Microsomes

Human 11β-HSD2 cDNA was amplified by RT-PCR and sub-cloned into pFastBac vector (Invitrogen). Recombinant baculovirus was generated using a Bac-to-Bac Baculovirus Expression System (Invitrogen). Sf9 insect cells infected with baculovirus were harvested 48 hours after infection and microsomes were prepared in the same way as described above.

11β-HSD2 Dehydrogenase Assay

The microsomes were incubated in reaction buffer for 20

minutes at room temperature (26°C) with 43 μM of NAD, respective concentrations of sterenins and 160 nM of cortisol. The reaction was terminated by the addition of 50 μM of carbenoxolone and the cortisol concentration was determined using HTRF Cortisol Assay Reagents.

Results

Taxonomy of the Producing Organism

The producing strain, SANK 21205, was isolated from the spore print of fresh basidiocarps collected in Gunma Prefecture, Japan. As this basidiomycete could not be characterized by its cultural features, the observation of dried basidiocarps was needed for identification. The morphological characteristics of the dried basidiocarps are shown in Fig. 2.

The basidiocarps were resupinate to effused-reflexed. The pilei were up to 5.0 mm in width, up to 0.4 mm in thickness, dimidiate to flabellate, and were usually coalescent and tough. The upper surface was hirsute and grayish white. The margin of the pilei was thin and acute. The hymenial surface was smooth to slightly tuberculate and pale orange. Cutis was present, which was brown and up to 25 μm in thickness.

The basidiospores were 8.0~12.0×3~4.0 μm, cylindrical, often slightly curved, thin-walled, smooth, and amyloid. The basidia were 40~50×5.0~6.0 μm, narrowly clavate, with 4 sterigamata, which were up to 4.0 μm in length. The pseudocystidia were 4.5~6.0 μm in width, cylindrical, thick-walled at the base (up to 2.0 μm), thin-walled at the apex, often with small points. The hyphal system was monomitic. The hyphae were 2.0~6.0 μm in width, thin-walled to thick-walled, and without clamp connections. Acanthohyphidia or pseudoacanthohyphidia were not observed.

The above characteristics of the basidiocarps corresponded with the description of the genus *Stereum* [8]. Hence, the strain was identified as *Stereum* sp. This strain has been deposited at the National Institute of Advanced Industrial Science and Technology, Japan, as *Stereum* sp. SANK 21205 under the accession number FERM BP-10465.

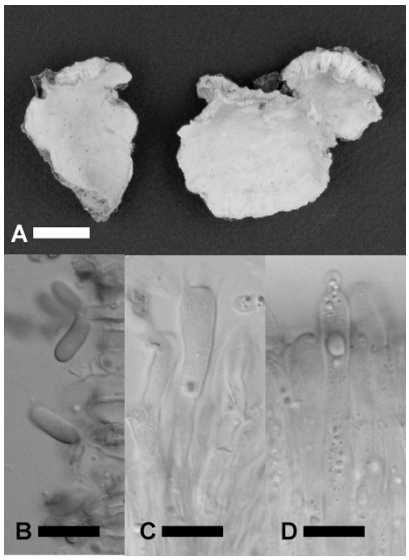

Fig. 2 Morphological characteristics of *Stereum* sp.

A: Basidiocarps. B: Basidiospores. C: Basidium. D: Pseudocystidium. Scales: A=10 mm; B~D=10 μm.

Fermentation

The growth of *Stereum* sp. SANK 21205 on an agar slant (1 cm^3) was homogenized with sterile water (3.0 ml). Then the whole amount of the homogenate was transferred into a 100-ml Erlenmeyer flask containing 30 ml of a sterilized liquid seed medium composed of glucose 0.4%, malt extract 1.0%, yeast extract 0.4%, agar 0.3% and Disfoam CB-442 (Nippon Yushi Co.) 0.005%. The flask was incubated at 23°C for 7 days on a rotary shaker at 210 rpm. The seed culture (30 ml) was transferred into a 500-ml AGRIFLEX bag (Fujimori Kogyo Co., Ltd.) containing a sterilized production medium which was composed of brown rice 90 g, $NaNO_3$ 0.05 g, KH_2PO_4 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.05 g and distilled water 100 ml. The fermentation was carried out at 23°C for 14 days by stationary cultivation.

Isolation

An equal volume of Me_2CO was added to the harvested culture (3.0 liters, 60 bags), which was left stirring for 30 minutes. And then, the extract was filtered by the aid of diatomaceous earth (Celite Co., 150 g). The yielded filtrate was adjusted to pH 3.0 with 6 M HCl and the active substance was extracted by EtOAc (3.5 liters). The extract was washed with saturated brine (2.0 liters) and dried over anhydrous Na_2SO_4 for 1 hour. The filtered extract was concentrated *in vacuo* to dryness to obtain oily material (13 g). The material was dissolved into MeOH (50 ml) and reversed phase silica gel powder (Cosmosil 140C_{18}-OPN, Nakalai Tesque, 60 g) was added to the solution by stirring. After the removal of the MeOH by evaporation, 0.3% triethylamine-phosphate buffer (pH 3.0, 400 ml) was added to the material adsorbed powder to make slurry. The obtained slurry was layered onto the Cosmosil 140C_{18}-OPN column (600 ml) equilibrated with 10% CH_3CN in H_2O containing 0.3% triethylamine-phosphate buffer (pH 3.0).

Table 1 Physico-chemical properties of sterenin A, B, C and D

	Sterenin A	Sterenin B	Sterenin C	Sterenin D
Appearance	White powder	White powder	White powder	White powder
Molecular formura	$C_{23}H_{25}NO_7$	$C_{26}H_{27}NO_{10}$	$C_{21}H_{21}NO_6$	$C_{23}H_{23}NO_8$
FAB-MS (*m/z*)	450 $(M+Na)^+$	514 $(M+H)^+$	406 $(M+Na)^+$	441 $(M+H)^+$
HRFAB-MS (*m/z*)	for $C_{23}H_{25}NO_7Na$	for $C_{26}H_{28}NO_{10}$	for $C_{21}H_{21}NO_6Na$	for $C_{23}H_{24}NO_8$
Found:	450.1526	514.1697	406.1273	442.1495
Calcd.:	450.1529	514.1713	406.1267	442.1502
$[\alpha]_D^{31}$	—	−13.6° (*c* 0.5, MeOH)	—	—
UV λ_{max}^{MeOH} nm (ε)	212 (58600), 265 (24400) 297 (10900)	214 (58100), 265 (26500) 296 (11100)	213 (66700), 268 (23000) 297 (12000)	213 (96200), 265 (41300) 297 (17500)
IR ν_{max}^{KBr} cm^{-1}	3357, 3231, 2973, 2931, 1662, 1623, 1602, 1456, 1376, 1311, 1255, 1199, 1164, 1084, 1064, 1035, 990	3348, 3264, 2973, 2928, 2858, 1718, 1662, 1624, 1603, 1450, 1413, 1377, 1310, 1254, 1198, 1162, 1087, 1063, 1035, 991	3409, 3267, 2974, 2931, 1669, 1653, 1623, 1599, 1504, 1446, 1413, 1374, 1349, 1313, 1255, 1193, 1173, 1161, 1100, 1063, 1038, 998, 989	3366, 3245, 2973, 2929, 2733, 1662, 1624, 1601, 1458, 1448, 1401, 1377, 1311, 1254, 1201, 1165, 1103, 1081, 1035, 995, 946
Solubility				
Soluble	EtOAc, MeOH, DMSO	EtOAc, MeOH, DMSO	EtOAc, MeOH, DMSO	EtOAc, MeOH, DMSO

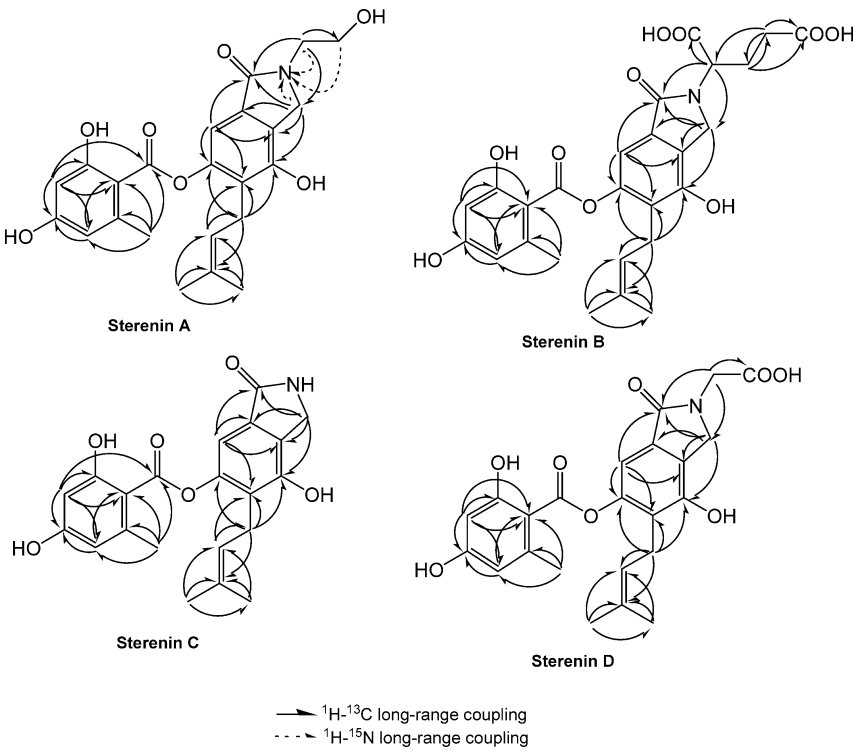

Fig. 3 ^{1}H-^{13}C and ^{1}H-^{15}N long-range couplings of sterenin A, B, C and D.

The column was washed successively with systems of the same solvent of 10, 20 and 25% (1.0 liter each) and the active material was eluted by 30% CH_3CN - H_2O containing 0.3% triethylamine-phosphate buffer (pH 3.0, 3.0 liters). The fraction which contained sterenin A, B, C and D (500 ml) was concentrated *in vacuo* to remove CH_3CN and

Table 2 NMR spectral data of sterenin A, B, C and D in CD_3OD

Position	Sterenin A			Sterenin B		Sterenin C		Sterenin D	
	δ_C (m[a])	δ_H (m, J in Hz)	δ_N	δ_C (m)	δ_H (m, *J* in Hz)	δ_C (m)	δ_H (m, *J* in Hz)	δ_C (m)	δ_H (m, *J* in Hz)
1	170.7 s			171.3 s		173.4 s		170.8 s	
2			123						
3	50.9 t	4.58 (2H, s)		46.9 t	4.46 (1H, d, 16.9)	45.0 t	4.40 (2H, s)	50.3 t	4.54 (2H, s)
					4.58 (1H, d, 16.9)				
3a	127.2 s			127.5 s[b]		129.3 s		127.5 s[b]	
4	152.4 s			152.4 s		152.6 s		152.4 s	
5	127.1 s			127.3 s[b]		127.3 s		127.3 s[b]	
6	151.3 s			151.4 s		151.4 s		151.4 s	
7	110.1 d	7.08 (1H, s)		110.4 d	7.09 (1H, s)	110.2 d	7.08 (1H, s)	110.4 d	7.08 (1H, s)
7a	132.7 s			132.0 s		132.5 s		132.1 s	
1′	24.7 t	3.37 (2H, m)		24.8 t	3.39 (2H, m)	24.7 t	3.38 (2H, m)	24.8 t	3.39 (2H, m)
2′	122.7 d	5.03 (1H, m)		122.7 d	5.04 (1H, m)	122.7 d	5.03 (1H, m)	122.7 d	5.04 (1H, m)
3′	133.3 s			133.3 s		133.3 s		133.3 s	
4′	17.9 q	1.50 (3H, s)		17.9 q	1.52 (3H, s)	17.9 q	1.50 (3H, s)	17.9 q	1.50 (3H, s)
5′	25.9 q	1.56 (3H, s)		25.9 q	1.57 (3H, s)	25.9 q	1.57 (3H, s)	25.9 q	1.57 (3H, s)
1″	105.3 s			105.3 s		105.3 s		105.3 s	
2″	167.1 s			167.1 s		167.1 s		167.1 s	
3″	102.0 d	6.23 (1H, br. s)		102.0 d	6.24 (1H, br. s)	102.0 d	6.22 (1H, br. s)	102.0 d	6.23 (1H, br. s)
4″	164.9 s			164.9 s		164.9 s		164.9 s	
5″	113.1 d	6.31 (1H, br. s)		113.1 d	6.31 (1H, br. s)	113.1 d	6.30 (1H, br. s)	113.1 d	6.30 (1H, br. s)
6″	145.1 s			145.1 s		145.1 s		145.1 s	
7″	171.8 s			171.8 s		171.8 s		171.8 s	
8″	24.7 q	2.60 (3H, s)		24.7 q	2.60 (3H, s)	24.7 q	2.60 (3H, s)	24.7 q	2.61 (3H, s)
1‴	46.5 t	3.75 (2H, t, 5.1)		55.5 d	4.99 (1H, m)			45.1 t	4.38 (2H, s)
2‴	61.3 t	3.83 (2H, br. t, 5.1)		26.3 t	2.25 (1H, m)			172.8 s	
					2.49 (1H, m)				
3‴				31.9 t	2.40 (2H, m)				
4‴				176.3 s					
5‴				173.9 s					

CD_3OD was used for an internal standard as δ_H 3.31 and δ_C 49.15. Urea was used for an external standard as δ_N 75. [a]: Multiplicity by DEPT experiment. [b]: Assignments are interchangeable.

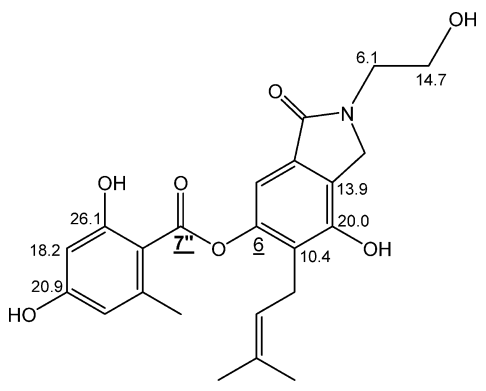

Fig. 4 Deuterium shift ($\Delta\delta_{CH_3OH-CD_3OD}$ in Hz) of sterenin A.

desalted by extraction with EtOAc (700 ml). The solvent layer was washed with saturated brine (600 ml) and dried over anhydrous Na_2SO_4 for 1 hour. Filtered extract was concentrated *in vacuo* to dryness to yield crude material (335 mg). Part of it (130 mg) was dissolved into MeOH (2.0 ml) and a 200 μl portion was applied to a preparative HPLC (column; Capcell Pak C18 UG120, 20×250 mm, mobile phase; CH_3CN - H_2O (37 : 63) containing 0.05% HCOOH, flow rate; 10 ml/minute, detection; UV at 210 nm). Preparations performed ten times obtained four isolated fractions which contained active compounds. Each fraction was concentrated *in vacuo* to remove the CH_3CN and, after freeze drying, they yielded pure sterenin A (24 mg), B (25 mg), C (11 mg) and D (10 mg) as white

powders.

Physico-chemical Properties

The physico-chemical properties of sterenin A, B, C and D are summarized in Table 1. The molecular formulae of these compounds were determined mainly by high-resolution FAB-MS. Sterenin A, B, C and D possess the molecular formulae of $C_{23}H_{25}NO_7$, $C_{26}H_{27}NO_{10}$, $C_{21}H_{21}NO_6$ and $C_{23}H_{23}NO_8$ based on their ions of $[M+Na]^+$, $[M+H]^+$, $[M+Na]^+$ and $[M+H]^+$, respectively. The ^{13}C- and ^1H-NMR spectral data of sterenin A, B, C and D observed in CD_3OD are shown in Table 2. These NMR spectral data suggest that their structures are closely related to each other.

Structure Elucidations

Sterenin A

The structure elucidation of sterenin A was carried out mainly by NMR spectral analysis. DEPT experiments revealed the presence of 3 methyl carbons, 4 methylene carbons, 4 methine carbons and 12 quaternary carbons. Assignments of the ^1H and ^{13}C signals were performed by

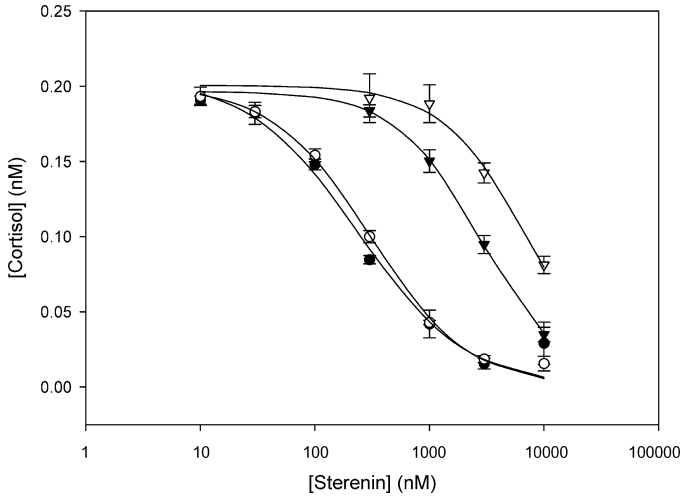

Fig. 5 Effects of sterenin derivatives on human 11β-HSD1 activity.

●: Sterenin A, ▽: sterenin B, ○: sterenin C, ▼: sterenin D.

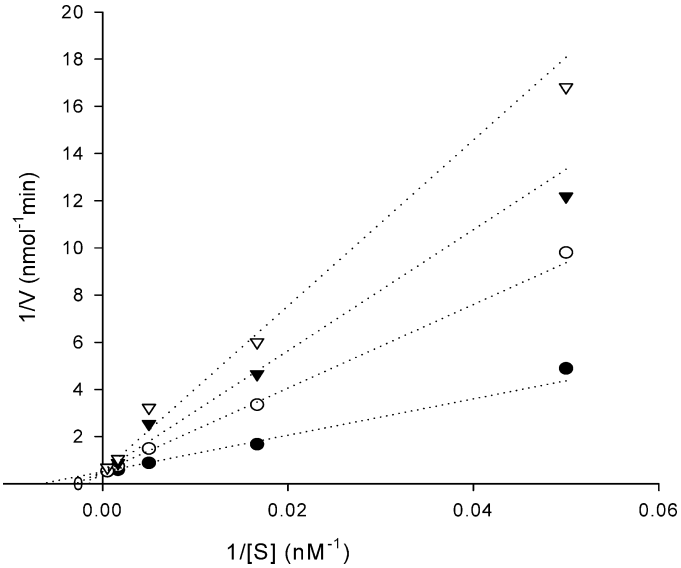

Fig. 6 Lineweaver-Burk plots for inhibition of human 11β-HSD1 by sterenin A.

● None, ○ 100 nM, ▼ 150 nM, ▽ 225 nM.

Table 3 Inhibitory activities of sterenin derivatives to human 11β-HSD isoforms

Sterenin	IC_{50} (nM)	
	11β-HSD1	11β-HSD2
A	240	>10000
B	6600	>10000
C	230	>10000
D	2600	>10000

detailed analysis of the DQF-COSY, HSQC and HMBC data. The observed ^{1}H-^{13}C and ^{1}H-^{15}N long-range couplings shown in Fig. 3 revealed isoprenylisoindolinone and *o*-orsellinic acid moieties.

The connectivity of these two partial structures was clarified by a deuterium shift experiment using samples in CD_3OD and in CD_3OH in a coaxial tube. The shift values in Hz ($\Delta\delta_{CH_3OH-CD_3OD}$) are shown in Fig. 4. All the carbon signals within the two-bond distance from the applicable oxygen atoms except for those around positions 6 and 7″ exhibited deuterium shifts and the isoprenylisoindolinone and *o*-orsellinic acid were thereby linked via an ester bond at C-6 and C-7″. Thus, the structure of sterenin A was elucidated as shown in Fig. 1.

Sterenin B, C and D

According to the NMR analysis of sterenin B, C and D shown in Fig. 3, it was concluded that they were different analogues based on variations of the *N*-linked side chain in sterenin A (Fig. 1).

Biological Activities

Sterenin homologues inhibited human 11β-HSD1 activity in a dose-dependent manner (Fig. 5). To analyse the 11β-HSD1 inhibition mechanism of sterenin A, we performed a kinetic analysis. The Lineweaver-Burk plots revealed that sterenin A behaved as a competitive inhibitor (Fig. 6). The *Ki* value of sterenin A was calculated to be 68 nM. The inhibitory effects of all the homologues were isoform specific, as they did not inhibit human 11β-HSD2 (Table 3).

Discussion

In the present study, we report the discovery of novel 11β-HSD1 inhibitors named sterenin A, B, C and D from a solid-state culture of producing basidiomycetes identified as *Stereum* sp. SANK 21205. Among various kinds of reported isoindolinone type alkaloids, 2,5-dimethyl-6-methoxy-4,7-dihydroisoindole-4,7-dione [9], described as the first naturally-occurring isoindole with antibacterial activity from sponge, stachybotrins [10] with antibacterial and fungicidal activities, staplabin [11] and a series of compounds named SMTPs [12～16] as tissue plasminogen modulators, are listed as related compounds. During the screening process, we isolated stachybotrin homologues, but their inhibitory activities against human 11β-HSD1 were approximately ten times weaker than those of sterenin A and C (data not shown). To the best of our knowledge, this is the first report of natural products which possess potent 11β-HSD1 inhibitory activities.

From the standpoint of the producing organism, there is literature that comments on *Stereum hirsutum*, which belongs to the same genus as the strain SANK 21205, and which produces a related compound named MS-3 [17～19]. Although this compound was reported to be a glyoxalase I inhibitor and a growth inhibitor of sarcoma cells *in vitro*, MS-3 would be one of the precursors of sterenin homologues in terms of biosynthesis.

As described above, sterenin derivatives are potent competitive inhibitors with strict selectivity against 11β-HSD1 versus 11β-HSD2. It is intriguing to note that sterenin A and C possess ten to thirty times stronger activities than those of sterenin B and D. Among these homologues, sterenin B exhibited the weakest inhibitory activity. In view of the contribution of its stereochemistry to the activity, we did not determine the configuration of sterenin B. It is possible to say that the size and/or charge of the *N*-linked side chain on the isoindolinone skeleton play a critical role in the inhibitory activity. Further structure-activity relationship (SAR) studies will be needed to determine the usefulness of sterenins against Type 2 diabetes and metabolic syndrome. The total synthesis of sterenins and the X-ray crystallographic analysis of their enzyme complexes will be separately reported elsewhere.

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