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Hirsutenols D, E and F, New Sesquiterpenes from the Culture Broth of *Stereum hirsutum*

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Abstract New sesquiterpenes, hirsutenols D∼F, were isolated from the fermentation broth of *Stereum hirsutum*, and their structures were determined on the basis of various spectroscopic analyses. Hirsutenols E and F showed significant scavenging activity against superoxide anion radicals with EC_{50} values of 1.62 and 0.39 mM, respectively.

Keywords *Stereum hirsutum*, hirsutenols, antioxidant, sesquiterpene

Free radicals have been known to be involved in various pathological processes represented by the pathogenesis of diseases such as myocardial and cerebral ischemia, atherosclerosis, diabetes, rheumatoid arthritis, cancer-initiation and aging processes [1∼3]. Thus, free radical scavengers are considered to be protective agents against these diseases. *Stereum hirsutum* is a producer of various tricyclic sesquiterpenes and we have reported the isolation of antioxidant substances hirsutenols A∼C [4] and sterins A∼C [5, 6] from the fermentation broth of this strain. Further investigation on the metabolites of *S. hirsutum* has resulted in isolation of new sesquiterpenes designated hirsutenols D (**1**), E (**2**) and F (**3**). We herein report the isolation, physico-chemical properties, structure determination and free radical scavenging activity of these compounds.

A strain of *S. hirsutum* grown on potato dextrose agar medium was used to inoculate a 500 ml Erlenmeyer flask containing 100 ml of the seed medium consisting of yeast extract 0.4%, malt extract 1.0% and glucose 0.4% (pH 6.0 before sterilization). The flask was shaken on a rotary shaker for four days at 27°C. The seed culture was transferred into a 5-liter jar fermenter containing 3 liters of the above medium for production of hirsutenols, and cultivation was carried out at 28°C for 12 days with aeration of 2 liters/minute and agitation of 250 rpm.

The fermentation broth (30 liters) was separated into supernatant and mycelia by centrifugation. The supernatant was applied to a Diaion HP-20 column, and the column was washed with 30% aqueous MeOH and eluted with 70% aqueous MeOH. After evaporation of the 70% aqueous MeOH eluent *in vacuo*, the resultant residue was partitioned between ethyl acetate and water. The EtOAc-soluble portion was subjected to a column of silica gel and eluted with $CHCl_3$: MeOH (30 : 1∼1 : 1, stepwise) to give active fractions. The antioxidative fractions were concentrated and applied to a Sephadex LH-20 column, which was developed with MeOH. The eluate was further purified by reverse-phase HPLC with ODS column (YMC, 250×20 mm; flow rate, 7 ml/minute) eluting with 15% aqueous MeOH to give **1** (r.t. 27 minutes, 11.1 mg) and eluting with 25% aqueous acetonitrile to afford **2** (r.t. 42 minutes, 1.2 mg) and **3** (r.t. 14 minutes, 4.1 mg).

The physico-chemical properties of hirsutenols D, E and F are summarized in Table 1, and ^{1}H and ^{13}C NMR spectral

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Table 1 Physico-chemical properties of hirsutenols D (**1**), E (**2**) and F (**3**)

	1	**2**	**3**
Appearance	Yellow powder	Yellow powder	Yellow powder
$[\alpha]_D^{20}$	+12.5 (*c* 0.09, MeOH)	+11 (*c* 0.09, MeOH)	−30 (*c* 0.1, MeOH)
Molecular formular	$C_{15}H_{18}O_5$	$C_{15}H_{24}O_3$	$C_{15}H_{18}O_4S$
Molecular weight	278	252	294
HR-FAB-MS *m/z*			
Found	301.1057 (M+Na$^+$)		295.1001 (M+H$^+$)
Calcd.	301.1052		295.1004
HR-ESI-MS *m/z*			
Found		275.1655 (M+Na$^+$)	
Calcd.		275.1623	
UVλ_{max} nm (in MeOH)	234 (ε 2.83)	221 (ε 2.89)	245 (ε 3.19)
IR ν_{max} cm^{-1} (KBr)	3368, 2926, 2855, 1661, 1567	3371, 2926, 2856, 1663, 1564, 1102	3369, 2927, 1746, 1691
TLC (Rf values)[a]	0.44	0.45	0.48
HPLC Rt (min)[b]	7.27	16.58	5.03
Soluble	MeOH, DMSO	MeOH, DMSO	MeOH, DMSO
Insoluble	Hexane, water	Hexane, water	Hexane, water

[a] Silica gel plate (Silica gel 60 F254, Merck), chloroform - methanol (7 : 1).
[b] YMC 4γ ODS, 4.6×150 mm, solvent: 15% MeOH for hirsutenol D, 25% CH_3CN for hirsutenols E and F.

Table 2 ^1H and ^{13}C NMR spectral data of hirsutenols D (**1**), E (**2**) and F (**3**) in CD_3OD

Canbon No.	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	76.7	4.60 (1H, s)	45.5	1.23 (1H, m) 1.94 (1H, m)	76.3	4.59 (1H, d, *J*=2.4 Hz)
2	185.9		49.8	2.61 (1H, dd, *J*=9.6, 19.2 Hz)	181.2	
3	58.6		61.1	1.46 (1H, d, *J*=7.2 Hz)	56.4	
4	149.5		80.0	3.76 (1H, dd, *J*=7.2 Hz)	60.9	2.62 (1H, br dd, *J*=3.6, 2.4 Hz)
5	205.2		66.9	3.27 (1H, m)	216.0	
6			69.6		45.9	2.80 (2H, s)
7	87.7		55.8		93.3	
8	72.4	4.44 (1H, s)	42.9	1.35 (1H, m) 1.75 (1H, dd, *J*=10.2, 13.8 Hz)	49.2	3.55 (1H, br s)
9	145.0		46.1	2.82 (1H, m)	144.0	
10	208.5		46.4	1.25 (1H, m) 1.95 (1H, m)	206.9	
11	54.8		48.5		55.8	
12	20.3	1.02 (3H, s)	68.7	3.26 (2H, m)	21.5	1.11 (3H, s)
13	23.8	1.11 (3H, s)	24.4	1.04 (3H, s)	23.7	1.21 (3H, s)
14	21.1	1.52 (3H, s)	13.4	1.28 (3H, s)	13.6	1.23 (3H, s)
15	122.4	5.72 (1H, s) 6.23 (1H, s)	22.0	1.32 (3H, s)	26.7	2.37 (1H, dd, *J*=3.6, 12.6 Hz) 2.59 (1H, dd, *J*=2.4, 12.6 Hz)

Chemical shifts in ppm from TMS as internal standard.
^1H and ^{13}C NMR were measured at 600 MHz and 150 MHz, respectively.

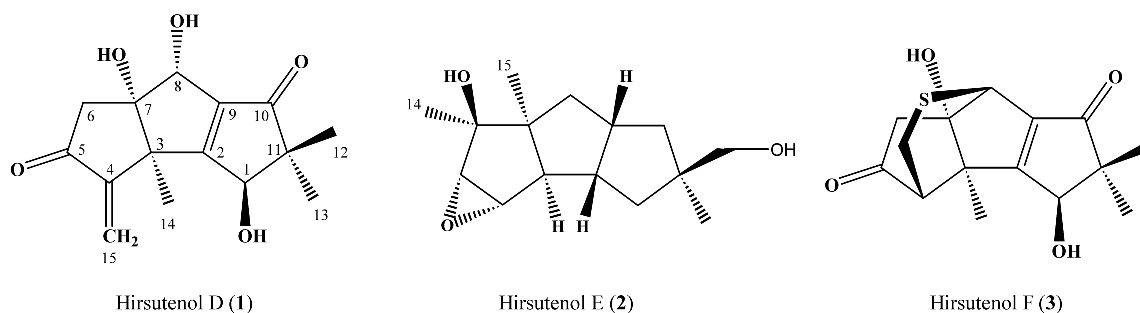

Fig. 1 Structures of hirsutenols D (**1**), E (**2**) and F (**3**).

data are in Table 2. The molecular formula of **1** was determined to be $C_{15}H_{18}O_5$ on the basis of high-resolution FAB-MS data [*m/z* found 301.1057 $(M+Na)^+$, calcd 301.1052]. The ^{1}H NMR spectrum of **1** in CD_3OD exhibited signals due to a terminal methylene at 6.23 and 5.72 ppm, two oxygenated methines at 4.60 and 4.44 ppm and three singlet methyls at 1.52, 1.11 and 1.02 ppm. In the ^{13}C NMR spectrum, 14 carbon peaks were observed. Direct ^{1}H-^{13}C connectivity was established by the HMQC experiment and chemical structure was assigned by interpretation of HMBC spectrum. HMBC showed long-range correlations from H-1 to C-2, C-9 and C-10, from H-8 to C-2, C-3 and C-9, from H-12 and H-13 to C-1, C-10 and C-11, from H-14 to C-2, C-3, C-4 and C-7, and from H-15 to C-4 and C-5. The chemical shift value of C-2 (185.9 ppm) suggested that a remained carbonyl carbon at 208.5 ppm should be placed at C-10 position, which was confirmed by HMBC cross-peaks from H-12 and H-13 to C-10. The above NMR data implied that hirsutenol D was structurally similar to hirsutenol A [4], and thus, the structure of **1** was established by the process of elimination in comparison with hirsutenol A. During the process of elimination, we found that a methylene signal of C-6 was not observed in both proton and carbon NMR spectra and it would seem to be quenched by deuterium exchange in CD_3OD. It is known that hydrogens adjacent to carbonyl carbon are sometimes quenched and this phenomenon was observed in the case of hirsutenol A. It could be confirmed by ^{1}H-NMR measurement in DMSO-d_6 after re-exchange of exchangeable protons in methanol. The ^{1}H NMR spectrum that was measured after re-exchange gave an additional methylene proton signal to that measured in CD_3OD. Therefore, the structure of **1** was determined as shown in Fig. 1. The relative stereochemistry was determined by the comparison with that of known tricyclic sesquiterpenes and by the aid of NOESY spectrum, which showed NOEs between H-1, H-13 and H-14. Also no NOEs between H-8 and H-1 and between H-8 and H-14 suggested that H-8 would be placed on anti-plane of H-1 and H-14.

The molecular formula of **2** was established as $C_{15}H_{24}O_3$ by high-resolution ESI-MS measurement. The ^{1}H NMR spectrum of **2** showed signals attributable to two oxygenated methines at 3.76 and 3.27 ppm, four methylenes including one oxygenated methylene, three methines at 2.61, 1.46 and 2.82 ppm and three methyls at 1.32, 1.28 and 1.04 ppm, while 15 carbons in the ^{13}C NMR spectrum were observed. Direct ^{1}H-^{13}C connectivity of these signals was established with the aid of an HMQC spectrum, and the structure was assigned by the HMBC spectrum. HMBC showed the critical long-range correlations from H-12 and H-13 to carbons of C-1, C-10, and C-11, from H-1 to C-9 and C-10, from H-2 to C-1, C-4 and C-7, from H-3 to C-1, C-4, C-7 and C-9, from H-4 to C-2, C-3 and C-5, from H-5 to C-3 and C-4, from H-8 to C-6, C-7, C-9 and C-10, from H-14 to C-5, C-6 and C-7 and from H-15 to C-3, C-6, C-7 and C-8. Thus, compound **2** was assigned as a new tricyclic sesquiterpene with an epoxy moiety. The epoxyl group was proposed by large $^1J_{CH}$ coupling constant of 185 Hz at C-4. The stereochemistry was proposed by the NOESY spectrum, which exhibited the NOE correlations between H-2, H-4, H-9 and H-1, and no NOEs between H-4 and H-14, H-4 and H-15, H-5 and H-14 and H-5 and H-15, implying that H-14 and H-15 should be placed on the anti-plane of H-2, H-4, H-5 and H-9.

The molecular formula of **3** was determined to be $C_{15}H_{18}O_4S_1$ by high-resolution FAB-MS data [*m/z* found 295.1001 $(M+H)^+$, calcd 295.1004], and IR absorptions at 3369 and 1746 cm^{-1} indicated the presence of hydroxyl and carbonyl groups. The ^{1}H and ^{13}C NMR spectra in the aid of the DEPT spectrum suggested that **3** was composed of two carbonyl carbons, two sp^2 quaternary carbons, three sp^3 quaternary carbons, three methines, two methylenes and three methyls. A partial structure –CH_2–CH– was established by the interpretation of proton coupling constants, and plenary structure was determined by the HMBC experiment in combination with the HMQC spectrum. HMBC correlations from H-1 to C-2, C-9 and C-10, from H-4 to C-3 and C-5, from H-6 to C-3, C-5, C-7

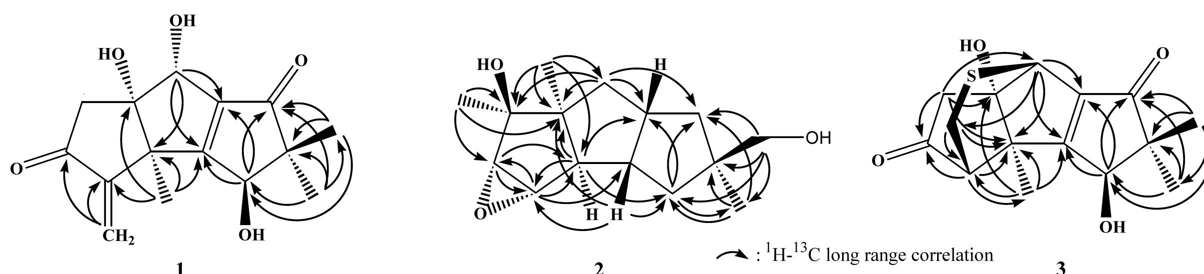

Fig. 2 HMBC correlations of hirsutenols D (**1**), E (**2**) and F (**3**).

and C-8, from H-8 to C-2, C-3, C-6, C-9 and C-15, and from H-15 to C-3, C-4 and C-8 established the structure of **3**, as shown in Fig. 2. The relative stereochemistry was determined by a NOESY spectrum, which exhibited cross-peaks between H-1 and H-13, H-4 and H-14, and H-12 and H-15.

Superoxide radical scavenging activity was assayed by the irradiated riboflavin/EDTA/NBT system with minor modifications [7]. The mixture consisted of 140 μl of 0.03 mM riboflavin, 1 mM EDTA, 0.6 mM methionine and 0.03 mM NBT solution in 50 mM potassium phosphate buffer (pH 7.8) and 10 μl of sample solutions, which include the test compounds and the reference compounds (α-tocopherol and trolox) at various concentrations in MeOH, as well as MeOH as a control. The photoinduced reactions to generate superoxide anion were carried out in an aluminum foil-lined box with two 20 W fluorescent lamps. The distance between reactant and lamp was adjusted until the intensity of illumination reached 1000 lux. The reactant was illuminated at 25°C for 7 minutes. The photochemically reduced riboflavin generated superoxide anion, which reduced NBT to blue formazan. Reduction of NBT was measured by the absorbance change at 570 nm before and after irradiation using a microplate. Hirsutenol F among the compounds tested exhibited the most potent superoxide radical scavenging activity with an EC_{50} of 0.39 mM, which was higher than α-tocopherol (0.71 mM) and trolox (1.56 mM) used as control. Hirsutenol E also showed significant scavenging activity against the superoxide radical anion with an EC_{50} value of 1.62 mM, while hirsutenol D displayed marginal activity with an EC_{50} value of 8.78 mM.

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