TERPESTACIN, A NEW SYNCYTIUM FORMATION INHIBITOR FROM *Arthrinium* sp.

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Terpestacin, a new antibiotic which inhibits syncytium formation, was isolated from *Arthrinium* sp. FA1744 (ATCC 74132). The structure of terpestacin was elucidated as a bicyclic sesterterpene on the basis of spectroscopic data and chemical derivatization.

The search for drugs to cure acquired immune deficiency syndrome (AIDS) is now considered to be one of the most important and challenging tasks for researchers in the pharmaceutical area. To date, the only approved anti-HIV drugs are nucleoside analogs, such as AZT and DDI. It is therefore of importance to identify other potential targets for the development of anti-HIV drugs. Human Immunodeficiency Virus (HIV), the causative agent of AIDS, is known to kill human T4 cells by invasion and budding resulting in cell membrane damage. In addition, the infected cells merge with healthy T4 cells *via* gp 120 expressed on cell surfaces, producing massive bodies consisting of many merged cells. These giant cells called syncytia can not survive, and are an indirect but major cause of death of T4 cells in HIV infection.[1)]

In the course of screening, we found that a fungal strain, FA1744, produced a new syncytium formation inhibitor, designated terpestacin.

This report deals with the taxonomy and fermentation of the producing organism, as well as isolation and characterization of terpestacin including determination of its planar structure and activity. Absolute structural elucidation and biosynthetic studies will be reported elsewhere.

Materials and Methods

Isolation of the Microorganism

Strain FA1744 was isolated from a soil sample collected in Akiyoshidoh, Yamaguchi prefecture, Japan.

Fermentation

A loopful of a malt extract agar culture of strain FA1744 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium. The medium consisted of soluble starch (Nichiden Kagaku) 2.0%, glucose 0.5%, NZ-case (Humko Scheffield) 0.3%, yeast extract (Oriental Yeast) 0.2%, fish meal D30X (Banyu Eiyo) 0.5% and $CaCO_3$ 0.3% prepared in tap-water. After formulation, its pH was adjusted to 7.0 with 1 N NaOH and then it was autoclaved at 121°C for 20 minutes. The seed flask was incubated for 4 days at 28°C on a rotary shaker (200 rpm). Five ml of this culture were transferred into a 500-ml

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Erlenmeyer flask containing 100 ml of the production medium which consisted of mashed potato (Snow Brand Milk Co.) 1.0%, glucose 0.3%, corn meal (Sakura Meal Co.) 2.0%, cane molasses (Nihon Tensaitoh) 1.0%, fish meal (Hokuyo Suisan) 0.5%, wheat bran 1.0%, NaCl 0.3% and $CaCO_3$ 0.3% prepared in tap-water (pH 7.0, before sterilization). The production fermentation was carried out at 28°C for 6 days on a rotary shaker (200 rpm).

The titer of terpestacin in the fermentation broth was determined by the syncytium formation inhibitory assay to be described later. After 6 days of fermentation, production reached a maximum titer of 35 μg/ml.

Syncytium Formation Inhibition Assay

The assay system for anti-syncytium formation agents consisted of two cell lines: BSC-1 cells infected with recombinant vaccinia virus expressing HIV-1 envelope glycoproteins gp 120 and gp 41, and HeLa-T4 cells expressing CD4 antigen. Both tester cell lines were mixed together in the presence and absence of an inhibitor and the number of syncytia formed in 3~5 hours was determined.

CD4-bearing HeLa (HeLa-T4) cells[2] were grown in DULBECCO's modified EAGLE's medium (D-MEM, GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO) and 1 mg/ml geneticin (GIBCO). BSC-1 cells were grown in EAGLE's MEM (E-MEM, Nissui Pharmaceutical, Tokyo) supplemented with 10% FBS and 50 μg/ml amikacin (Bristol-Myers Squibb). A monolayer culture of BSC-1 cells (3-day-old) in a T-75 LUX flask (Sanko Junyaku, Tokyo) was inoculated with recombinant vaccinia virus (v-*env*5)[3] at a multiplicity of infection of 0.01. After viral adsorption for one hour, the inoculum was removed by aspiration. The virus-infected BSC-1 cells were resuspended in 8 ml of fresh E-MEM and were incubated at 37°C for an additional 20 to 24 hours in a humidified environment consisting of 5% CO_2 and 95% air. After the infected cells were removed from the plastic flask surface with a cell scraper, the syncytium formation titer of the HIV-1 *env*-expressing BSC-1 cells was determined by adding aliquots of cell suspension at 2-fold serial dilutions to HeLa-T4 cells and measuring the number of syncytia formed. The syncytium formation titer of the cell suspension was adjusted 6×10^4 syncytia per milliliter. A HeLa cell suspension (100 μl containing 3×10^4 cells) was seeded into each well of a 96-well microtiter plate and was incubated at 37°C for 20 to 24 hours in the humidified environment. After incubation, the original medium was removed and replaced by 50 μl of fresh E-MEM supplemented with 10% FBS which contained a test sample at various concentrations. Then the HIV-1 *env*-expressing BSC-1 cell suspension was diluted with E-MEM at 1:15. Fifty μl of the dilution was added to each well of the 96-well microtiter plate. After incubation at 37°C for 3~5 hours, the medium in each well was removed by aspiration. Cells were stained with 50 μl Giemsa solution (Wako Pure Chemicals) and were washed 3 times with tap-water. The number of syncytia in each well was scored using light microscopy at a magnification of 40. Syncytium formation inhibitory activity of a test compound was expressed as the ID_{50} (50% inhibitory dose) which was defined as the minimal concentration of a compound required to reduce syncytium formation by 50% as compared to the inhibitor-untreated control.

Instrumental Analyses

The IR and UV spectra were recorded on a JASCO IR-810 IR spectrophotometer and a JASCO UVIDEC-610C spectrometer, respectively. The ^1H and ^{13}C NMR spectra were determined on a JEOL JNM-GX 400. The MS spectra were recorded on a JEOL JMS-AX505H mass spectrometer.

Terpestacin Triacetate Preparation

A mixture of terpestacin (30 mg), pyridine (1 ml) and acetic anhydride (1 ml) was stirred for 3 hours at room temperature. The mixture was diluted with methylene chloride (10 ml) and the organic layer was washed with water (1 ml), dried over $MgSO_4$ and concentrated to dryness. The resulting oil was chromatographed on a Sephadex LH-20 column which was eluted with methylene chloride-methanol (1:1) to give pure terpestacin triacetate as an oil (31 mg): IR (KBr) cm^{-1} 1780, 1720; UV (MeOH) λ_{max} nm 235 (ε 8,600); FAB-MS *m/z* 551 $(M+Na)^+$, 529 $(M+H)^+$; HRFAB-MS calcd for $C_{31}H_{45}O_7$ $(M+H)^+$ 529.3165, found 529.3133.

Hexahydro Terpestacin Preparation

A solution of terpestacin (50 mg) in methanol (5 ml) was hydrogenated using 10% palladium on charcoal (2 mg) under atmospheric pressure for 12 hours. After the mixture was filtered, the filtrate was

concentrated. The resulting oil was purified using preparative HPLC (column; YMC-301-3 Yamamura Chem. Lab. Co., eluant; 55% aqueous acetonitrile) to yield the title compound as an amorphous powder (6.7 mg): MP > 69°C; IR (KBr) cm^{-1} 1700, 1650; UV (MeOH) λ_{max} nm 264 (ε 11,000); ^1H NMR (DMSO-d_6) δ 0.79 (3H, d, J=6.8 Hz), 0.86 (3H, d, J=6.4 Hz), 0.87 (3H, d, J=6.8 Hz), 0.96 (3H, s), 1.15 (3H, d, J=6.8 Hz), 3.51 (1H, dd, J=7.5 and 10.3 Hz), 3.55 (1H, dd, J=6.4 and 10.3 Hz), 4.18 (1H, s, exchangeable with D_2O), 4.25 (1H, d, J=4.7 Hz, exchangeable with D_2O); FAB-MS m/z 431 $(M+Na)^+$, 409 $(M+H)^+$; HRFAB-MS calcd for $C_{25}H_{45}O_4$ $(M+H)^+$ 409.3318, found 409.3315.

Results and Discussion

Macroscopic and Microscopic Observations

Strain FA1744 showed good growth on potato-dextrose, potato starch, corn meal and malt extract agars. On malt extract agar, this strain formed rather rapidly growing and grayish olive colonies, attaining 80~85 mm in diameter after incubation for 14 days at 25°C. The colony surface was thick and floccose. The colony reverse was light yellowish brown to dark grayish brown. No diffusible pigment was produced. Conidial structures were produced abundantly on the surface. A sexual reproductive organ was not observed.

Conidiophores arose from conidiophore mother cells which were subspherical or ampulliform-shaped, and 5~8×3~5 μm in size. The conidiophores were hyaline and refractive, measuring more than 30 μm in length and 1.5~3 μm in thickness with brown transverse septa. Conidia were dark brown, lenticular-shaped with a colorless band at the junction of the two sides and were 7~9 μm in diameter (Fig. 1).

Physiological Characteristics

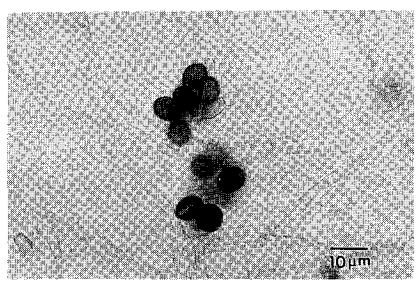
Strain FA1744 showed good growth at 10~30°C on malt extract agar. The optimum temperature for growth was 20~25°C.

On the basis of these morphological observations, strain FA1744 was assigned to the hyphomycetal genus *Arthrinium* Kunze, according to the description of ELLIS[4,5] and was thus identified as a species of *Arthrinium*. This strain was deposited in the American Type Culture Collection with the accession number of ATCC 74132.

Product Isolation

The fermentation beer (9 liters) was filtered with diatomaceous earth. The mycelial cake was extracted twice with methanol (2×1 liters), and the combined extracts were concentrated *in vacuo* to about 200 ml. The concentrate was extracted with ethyl acetate (200 ml), and the extract evaporated to give 3.6 g of a brown oil. The oil was chromatographed on a silica gel column (E. Merck Kieselgel 60, 500 ml) using methylene chloride (1 liter) and then with methylene chloride-methanol (95:5, 2 liters). The eluate was collected in 20 g-fractions; and each fraction was monitored by HPLC (YMC A-301-2 Yamamura Chem. Lab. Co., eluant; 50% aqueous acetonitrile, flow rate; 1.2 ml/minute, detection; UV at 254 nm, retention time; 4.5 minutes) and by the syncytium inhibition assay. Evaporation of the collected active fractions (Nos. 46~63) yielded a yellow amorphous powder (1.84 g). The powder was dissolved in aqueous acetonitrile (20 ml) and was chromatographed on a reverse phase silica gel column (150

Fig. 1. Conidia of strain FA1744 on potato-dextrose agar (incubated for 14 days at 25°C).



ml, YMC-GEL ODS-A 60-350/250 Yamamura Chem. Lab. Co.). The column was developed with 30% aqueous acetonitrile (1 liter) and then with 50% aqueous acetonitrile (1.5 liters). Terpestacin was eluted with 50% aqueous acetonitrile and the appropriate eluate fractions were combined and evaporated to give 316 mg of a light-yellow powder. This semi-pure sample (310 mg) was further purified by Sephadex LH-20 (800 ml) column chromatography using methylene chloride-methanol (1:1). The appropriate fractions were combined and evaporated to give pure terpestacin as a colorless amorphous powder (263 mg). A sample (10 mg) was crystallized from aqueous methanol to yield monoclinic crystals (8 mg).

Physico-chemical Properties

Terpestacin was isolated as monoclinic crystals. It was soluble in dimethyl sulfoxide, methanol, methylene chloride, ethyl acetate and alkaline water (such as 0.1 N NaOH), but was insoluble in hexane and water. It gave positive responses to iodine vapor, sulfuric acid and ferric chloride, but negative responses to Rydon-Smith, Dragendorff and anthrone-sulfuric acid reagents on a silica gel TLC plate. Other physico-chemical properties of terpestacin are summarized in Table 1. The IR, ^{1}H NMR (Table 2) and ^{13}C NMR (Table 3) spectra of terpestacin are shown in Figs. 2, 3 and 4, respectively. The physico-chemical and spectrometric properties of terpestacin are different from those of any known antibiotics.

Table 1. Physico-chemical properties of terpestacin.

Nature	Colorless monoclinic crystals
MP	172~173°C
$[\alpha]_D^{22}$ (c 0.5, $CHCl_3$)	+26°
Molecular formula	$C_{25}H_{38}O_4$
HR-MS (m/z)	
Calcd for $C_{25}H_{38}O_4$	402.2752
Found	402.2761
Microanalysis	$C_{25}H_{38}O_4 \cdot H_2O$
Calcd for	C 71.39, H 9.59
Found	C 71.77, H 9.30
UV λ_{max} nm (ε)	264 (10,800) in MeOH, 298 (8,500) in alkaline MeOH (0.01 N NaOH)
IR ν (KBr) cm^{-1}	3350, 1690, 1645, 1450, 1405, 1040, 1020
HPLC[a] Rt (minutes)	4.5
TLC[b] Rf	0.41

[a] YMC A-301-3 Yamamura Chem. Lab. Co.; eluant, CH_3CN-H_2O, 1:1; flow rate 1.2 ml/minute; detection UV at 254 nm.
[b] Merck Kieselgel 60; CH_2Cl_2-CH_3OH, 10:1; detection by I_2.

Structural Studies

The molecular composition ($C_{25}H_{38}O_4$) of terpestacin showed the presence of 7° of unsaturation, 5° of which were attributable to a carbonyl

Fig. 2. IR spectrum of terpestacin (KBr disk).

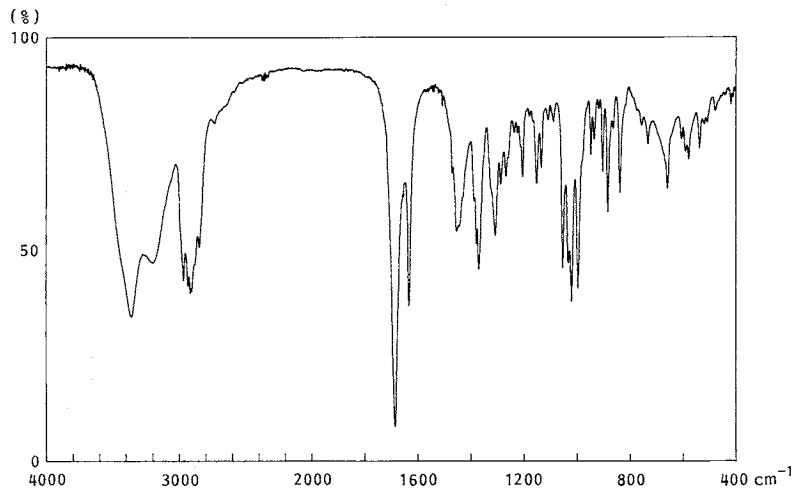


Fig. 3. ^{1}H NMR spectrum of terpestacin (400 MHz, DMSO-d_6).

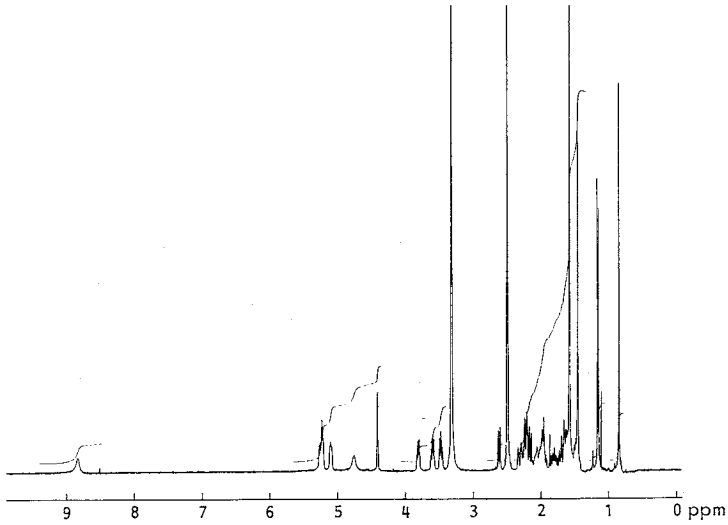
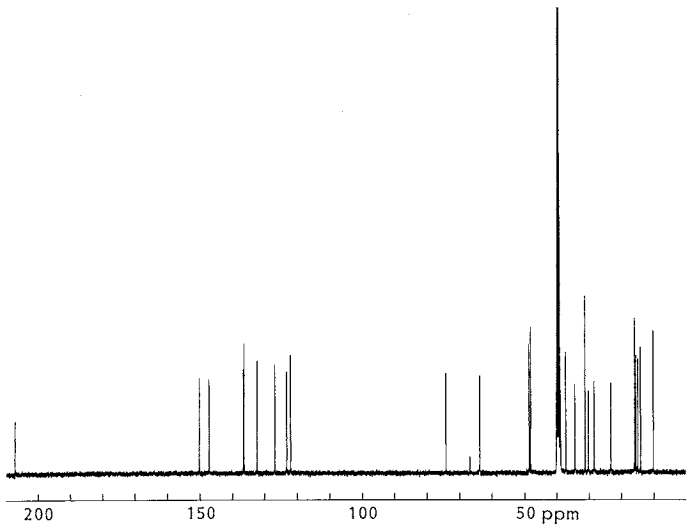


Fig. 4. ^{13}C NMR spectrum of terpestacin (100 MHz, DMSO-d_6).



group (δ 207.16) and four carbon-carbon double bonds (δ 150.26, 147.21, 136.70, 136.44, 132.38, 126.94, 123.28 and 122.09) based on ^{13}C NMR. The residual 2° suggested a possibility of a bicyclic structure for terpestacin.

The four oxygen atoms in terpestacin were assigned as one carbonyl and three hydroxyl groups based on ^{1}H NMR. Acetylation (Ac_2O/pyridine) of terpestacin produced a triacetate (EI-MS m/z 528 $(M)^+$), which confirmed this assignment. The positive reaction to ferric chloride suggested that at least one of the hydroxyl groups was enolic. The UV spectrum of the triacetate exhibited an absorption maximum at 235 nm in methanol. The 29 nm hypochromic shift by acetylation strongly indicated that the chromophore of terpestacin was a 3-alkyl-2-hydroxy-2-cyclopenten-1-one.[6] The spectral comparison (Table 4) of 2-hydroxy-3-methyl-2-cyclopenten-1-one[7] and the triacetate of terpestacin supported the presence of this chromophore.

Hydrogenation (H_2/Pd-C) of terpestacin gave a hexahydro derivative: EI-MS m/z 408; UV λ_{max} 264 nm (ε 11,100 in MeOH); IR ν (KBr) cm^{-1} 3400, 1700, 1650. In the ^1H NMR spectrum of the derivative, the three methyls resonated at higher fields (δ 0.79, 0.86 and 0.87) than those of terpestacin (δ 1.46, 1.58 and 1.58) and the three vinyl protons (δ 5.11, 5.22 and 5.26) of terpestacin were obviously absent.

The structural studies were carried out by 2D NMR experiments. The ^1H NMR and ^{13}C NMR data for terpestacin and its triacetate assigned by HETCOR and long range HETCOR are summarized in Tables 2 and 3. The ^1H-^1H COSY and HETCOR experiments identified a C25-methyl (3H, δ 1.16)–C23-methine (1H, δ 2.50)–C24-methylene (2H, δ 3.48 and 3.60) proton spin system associated with the C25 (δ 14.13, q), C23 (δ 37.21,

Table 2. ^1H NMR spectra of terpestacin and its triacetate (400 MHz, in DMSO-d_6).

Position	δ ppm (splitting, J=Hz)	
	Terpestacin	Terpestacin triacetate
2	1.68, 2.18 (m)	1.77, 2.17 (m)
3	5.26 (m)	5.29 (dd, 4.6, 9.5)
5	1.95, 2.23 (m)	1.96, 2.22 (m)
6	2.09, 2.23 (m)	2.11, 2.23 (m)
7	5.11 (m)	5.11 (m)
9	1.72, 1.98 (m)	1.75, 2.04 (m)
10	1.50 (m)	1.74 (m)
11	3.81 (m)	5.09 (m)
13	5.22 (m)	5.45 (m)
14	1.83, 2.32 (m)	1.95, 2.41 (m)
15	2.62 (br d, 9.8)	2.99 (dd, 9.2, 1.1)
19	0.85 (s)	0.90 (s)
20	1.58 (s)	1.58 (s)
21	1.58 (s)	1.60 (s)
22	1.46 (s)	1.55 (s)
23	2.50 (m)	2.94 (m)
24	3.48 (dd, 7.3, 9.8), 3.60 (dd, 7.2, 9.8)	4.05 (dd, 7.7, 10.8), 4.13 (dd, 6.1, 10.8)
25	1.16 (d, 6.9)	1.18 (d, 6.9)

Table 3. ^{13}C NMR spectra of terpestacin and its triacetate (100 MHz, in DMSO-d_6).

Position	δ ppm	
	Terpestacin	Terpestacin triacetate
1	48.52 (s)	48.96 (s)
2	38.93 (t)	38.40 (t)
3	122.09 (d)	121.27 (d)
4	136.44 (s)	137.09 (s)
5	39.78 (t)	39.65 (t)
6	23.27 (t)	23.35 (t)
7	123.28 (d)	123.70 (d)
8	132.38 (s)	132.05 (s)
9	34.39 (t)	34.04 (t)
10	30.22 (t)	27.14 (t)
11	74.19 (d)	77.83 (d)
12	136.70 (s)	131.85 (s)
13	126.94 (d)	130.84 (d)
14	28.45 (t)	28.00 (t)
15	48.07 (d)	48.71 (d)
16	150.26 (s)	164.14 (s)
17	147.21 (s)	144.27 (s)
18	207.16 (s)	204.13 (s)
19	15.97 (q)	15.53 (q)
20	15.00 (q)	14.94 (q)
21	15.58 (q)	15.25 (q)
22	10.21 (q)	10.85 (q)
23	37.21 (d)	33.21 (d)
24	63.77 (t)	65.83 (t)
25	14.13 (q)	14.36 (q)
3×$OCOCH_3$		19.96 (q), 20.35 (q), 20.98 (q), 167.04 (s), 169.32 (s), 170.01 (s)

Multiplicity was determined by DEPT data.

Table 4. Selected spectral data of terpestacin, its triacetate, 2-hydroxy-3-methyl-2-cyclopenten-1-one and 2-acetoxy-3-methyl-2-cyclopenten-1-one.

Compound	IR (C=O) cm^{-1}	UV nm (ε×10^{-3})		^{13}C NMR (δ ppm)[a]		
		MeOH	MeOH (OH^-)	C-1	C-2	C-3
Terpestacin	1700	264 (10.8)	298 (8.5)	207.16	147.21	150.26
Terpestacin triacetate	1780, 1720	235 (8.6)	—	204.13	144.27	164.14
2-Hydroxy-3-methyl-2-cyclopenten-1-one	1770	258 (10)	294 (7.1)	202.00	143.58	149.56
2-Acetoxy-3-methyl-2-cyclopenten-1-one	1775, 1705	232 (8.2)	—	199.45	145.23	161.50

[a] C-1, C-2 and C-3: Carbonyl, α and β carbons of the cyclopenten-1-one.

Fig. 5. ^{1}H-^{13}C long range connectivities of terpestacin and partial structures A, B and C.

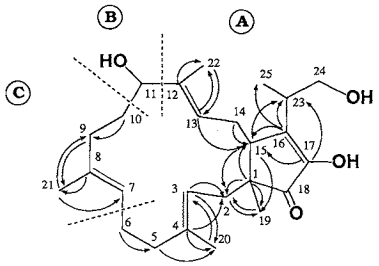


Table 5. Comparative syncytium formation inhibitory activities of terpestacin and dextran sulfate.

Compound	ID_{50} (μg/ml)
Terpestacin	0.46
Dextran sulfate	12.0

d) and C24 (δ 63.77, t) carbon signals. The ^{13}C chemical shift was indicative of attachment of a hydroxyl group at C24. Long range HETCOR experiments showed that C23 was connected with C16 (δ 150.26) of the cyclopentenone ring, which in turn showed a cross peak with C15-methine proton (δ 2.62). Extensive ^{1}H-^{1}H COSY and long range HETCOR experiments established the three partial structures A, B and C shown in Fig. 5. The sequence of these three units, however, could not be determined from the NMR data because of the overlap of the C22-methyl and C10-methylene protons and the absence of a diagnostic cross peak between H6 and H7. To unambiguously establish the skeleton of terpestacin, 2D-INADEQUATE experiments were carried out for triacetyl-terpestacin which exhibited improved solubility and well separated ^{13}C NMR signals in DMSO-d_6. These data clearly showed the connectivity between C10 and C11, C11 and C12 and C6 and C7 establishing the skeleton of the triacetate.

The planar structure of terpestacin was determined on the basis of these results to be as shown in Fig. 5. Its absolute structure and biosynthetic pathway will be reported elsewhere.

Biological Properties

Dextran sulfate was used as the reference compound for syncytium formation inhibitory activity. The results reported in Table 5 showed that terpestacin had more potent syncytium formation inhibitory activity (ID_{50}: 0.46 μg/ml) than dextran sulfate (ID_{50}: 12.0 μg/ml).

Terpestacin had weak antimicrobial activity against *Staphylococcus aureus* FDA 209P and *Staphylococcus aureus* Smith with MIC values of 25 μg/ml and 100 μg/ml, respectively, but no activity at 100 μg/ml against *Escherichia coli* Juhl, *Klebsiella pneumoniae* PCI 602 and *Proteus vulgaris* IMP-13.

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