# WF11899A, B AND C, NOVEL ANTIFUNGAL LIPOPEPTIDES

## I. TAXONOMY, FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES

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WF11899A, B and C, novel antifungal lipopeptide antibiotics were isolated from the culture broth of *Coleophoma empetri* F-11899. These compounds belong to the echinocandin type of lipopeptides. Of these compounds, WF11899A showed good solubility in water. These three antibiotics possess potent *in vitro* antifungal activities against *Candida* spp.

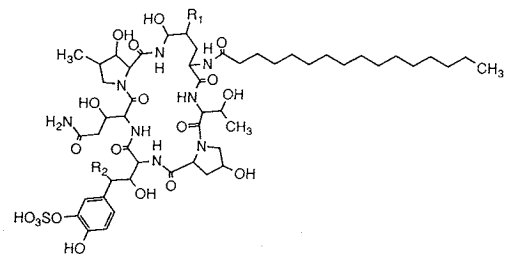
Echinocandin B[1] is an antifungal lipopeptide antibiotic which is structurally characterized by a cyclic hexapeptide acylated with a long side chain . Other related lipopeptides from fungal origin are known as aculeacins[2], mulundocandin[3] and pneumocandins[4,5], which are produced by *Aspergillus aculeatus*, *A. sydowi* and *Zalerion arboricola*, respectively. These antibiotics have excellent anti-*Candida* activity attributed to selective inhibition of 1,3-$\beta$-glucan synthesis. Furthermore, pneumocandins were reported to be active against *Pneumocystis carinii*[6], pathogen causing pneumonia in AIDS patients. They are, however, barely soluble in water. This insolubility is one of the reasons why they cannot be developed as a clinical use.

In the course of our screening for new antifungal antibiotics, we found that a strain of *Coleophoma empetri* F-11899 produces water-soluble echinocandin analogs, WF11899A, B and C[7] (Fig. 1). This paper describes taxonomic studies on the producing strain, fermentation, isolation and physico-chemical properties of these compounds.

### Taxonomic Studies

The strain F-11899 was originally isolated from a soil sample collected at Iwaki-shi, Fukushima-ken, Japan. This organism grew rather restrictedly on various culture media, attaining 1.0~4.5 cm in diameter after two weeks at 25°C, and it formed dark grey to brownish grey colonies (Table 1). The morphological characteristics were determined on the basis of the cultures on sterilized azalea leaf affixed to a MIURA's LCA plate[9] because the strain produced conidial structures on the leaf segment alone (Fig. 2). Conidiomata were pycnidial, superficial, separate, discoid to ampulliform, flattened at the base, unilocular, thin-walled, black, 90~160 (~200) $\mu$m in diameter and 40~70 $\mu$m high. Ostiole was often single, circular, central, papillate, 10~30 $\mu$m in diameter and 10~20 $\mu$m high. Conidiophores formed from the lower layer of inner pycnidial walls. They

Fig. 1. Structures of WF11899A, B and C.



WF11899A $R_1$=OH, $R_2$=OH
WF11899B $R_1$=OH, $R_2$=H
WF11899C $R_1$=H, $R_2$=H

Table 1. Cultural characteristics of *C. empetri* F-11899.

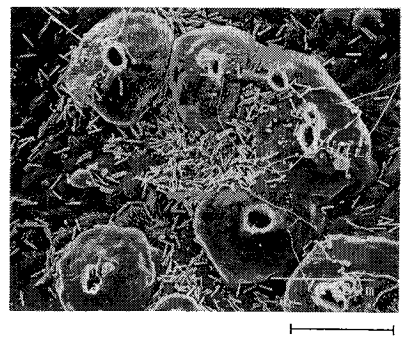
| Medium | Growth | Colony surface | Reverse |
|---|---|---|---|
| Malt extract agar | Rather restrictedly 2.5~3.0 cm | Circular, plane, thin to felty, olive brown (4F5)[a], arising aerial hyphae at the center (yellowish grey (4B2)) | Brownish grey (4F2) |
| Potato dextrose agar | Rather rapidly 3.5~4.0 cm | Circular, plane, felty, somewhat wrinkly, brownish grey (4F2), arising aerial hyphae at the center (pale grey (4B1) to brownish grey (4F2)) | Dark grey (4F1) |
| CZAPECK's solution agar | Very restrictedly 1.0~1.5 cm | Irregular, thin, scanty, immersed, subhyaline to white | Subhyaline to white |
| Sabouraud dextrose agar | Restrictedly 2.0~2.5 cm | Circular, plane, thin, white, sectoring, light brown (6D5) at the colony center | Pale yellow (4A3) |
| Oatmeal agar | Fairly rapidly 4.0~4.5 cm | Circular, plane, felty to cottony, dark grey (4F1) to brownish grey (4F2) | Brownish grey (4D2) |
| Emerson Yp Ss agar | Restrictedly 2.0~2.5 cm | Circular to irregular, plane, felty, dark grey (4F1) to brownish grey (4F2) | Medium grey (4E1) to dark grey (4F1) |
| Corn meal agar | Rather restrictedly 2.5~3.0 cm | Circular, plane, thin to felty, dark grey (2F1) to olive (2F3) | Dark grey (2F1) to olive (2F3) |
| MY20 agar | Restrictedly 1.5~2.0 cm | Circular to irregular, thin, sectoring, yellowish white (4A2) | Pale yellow (4A3) to orange white (5A2) |

[a] Color descriptions based on the Methuen Handbook of Colour[8].

were hyaline, simple or sparingly branched, septate and smooth. Conidiogenous cells were enteroblastic, phialidic, determinate, ampulliform to obpyriform, hyaline, smooth, 5~8×4~6 μm, with a collarette. The collarettes were campanulate to cylindrical and 14~18×3~5 μm. Conidia were hyaline, cylindrical, thin-walled, aseptate, smooth and 14~16 (~18)×2~3 μm. The vegetative hyphae were septate, brown, smooth and branched. The hyphal cells were cylindrical and 2~7 μm thick. The chlamydospores were absent. The strain F-11899 had an optimum temperature of 23 to 27°C on potato dextrose agar.

Fig. 2. Scanning electron micrograph of conidial structures of *C. empetri* F-11899 formed on an autoclaved azalea leaf placed on MIURA's LCA plate.

Bar represents 100 μm.

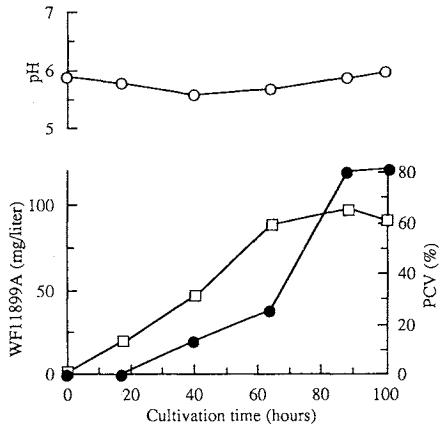


The above characteristics indicated that the strain F-11899 resembled *Coleophoma empetri* (Rostrup) Petrak 1929[10~12] belonging to the order Coelomycetes, but differed in some pycnidial characteristics: globose or flattened at the base, immersed, and not papillate. Therefore, the strain was formerly designated "Coelomycetes strain F-11899" or "*Coleophoma* sp. F-11899" in patent documents. However, the original description of *C. empetri* was made on the observations of conidial structures in living leaves without cultural studies, therefore, it was difficult to compare the pycnidial characteristics of the strain F-11899 with the description of *C. empetri* on the same conditions. Other morphological characteristics such as conidiophers, conidiogenous cells or conidia of the strain F-11899 and *C. empetri* agreed well.

The above differences are insufficient to justify their separation into independent species, because the pycnidial characteristics are variable on cultural condition (personal communication from Dr. B. C. SUTTON).

Consequently, the strain F-11899 was classified as a strain of *C. empetri* (Rostrup) Petrak 1929 and designated as *C. empetri* F-11899. The strain has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-2635.

Fig. 3. Time course of fermentation of strain F-11899.
● Potency, ○ pH, □ packed cell volume (PCV).




## Fermentation

A seed medium (160 ml) consisted of sucrose 1%, cotton seed meal 2%, dried yeast 1%, peptone 1%, $KH_2PO_4$ 0.2%, $CaCO_3$ 0.2% and Tween 80 0.1% was poured into each of two 500-ml Erlenmeyer flasks and sterilized at 121°C for 30 minutes. A loopful of the slant culture of *C. empetri* F-11899 was inoculated to each of the medium and cultured under shaking condition (250 rpm) at 25°C for 90 hours.

A production medium (20 liters) consisted of corn starch 3%, glucose 1%, wheat germ 0.2%, cotton seed meal 2%, gluten meal 0.5%, $KH_2PO_4$ 2%, $Na_2HPO_4 \cdot 12H_2O$ 1.5%, $ZnSO_4 \cdot 7H_2O$ 0.001% and Adekanol LG-109 (antifoam) 0.05% was poured into a 30-liter jar fermentor and sterilized at 121°C for 30 minutes.

The resultant seed cultured broth (320 ml) was transferred to the production medium. Fermentation was carried out at 25°C for 90 hours with aeration at 20 liters per minute. The agitation was 250 rpm and up to 400 rpm at 40 hours after inoculation. A typical time course of WF11899A production is shown in Fig. 3.

## Isolation

The culture broth (20 liters) was extracted with an equal volume of acetone by stirring for 2 hours at room temperature. The extract was filtered through diatomaceous earth and the filtrate was concentrated *in vacuo* to remove acetone. The resultant aqueous solution was washed twice with EtOAc (2 × 10 liters) to separate lipophilic contaminants, and anti-fungal activity was then extracted into *n*-BuOH (2 × 10 liters). The *n*-BuOH extracts were combined and concentrated to an oil. The oily materials were coated with silica gel, and it was applied to a column (300 ml) of silica gel (Kiesel gel 60, E. Merck) and the column was eluted with mixtures of $CH_2Cl_2$-MeOH. The active fractions eluted with $CH_2Cl_2$-MeOH (3 : 1 ~ 1 : 1) were concentrated *in vacuo* to dryness. The residue was dissolved in 50% aqueous MeOH and subjected to an ODS silica gel (YMC GEL ODS-AM, YMC Co., Ltd. 250 ml) column chromatography. The column was washed with 50% aqueous MeOH and the active principles were eluted with 80% aqueous MeOH. The eluate was concentrated and was further purified on a centrifugal partition chromatography (CPC) using a solvent system *n*-BuOH - MeOH - $H_2O$ (4 : 1 : 5) of upper stationary phase and lower mobile phase in a descending mode. The objective fractions containing WF11899A, which was eluted prior to WF11899B and C, were combined, concentrated *in vacuo* and applied to a column (35 ml) of silica gel. The column was developed with *n*-BuOH - AcOH - $H_2O$ (6 : 1 : 1). The fractions containing WF11899A were concentrated to dryness after adjusted to pH 5.0, and dissolved in a small volume of 50% aqueous MeOH. The solution

was passed through a column (3.5 ml) of ODS-AM. WF11899A was eluted with MeOH after washing the column with 50% aqueous MeOH. The eluate was concentrated to dryness, dissolved in water and adjusted to pH 7.0 with 0.01N NaOH. The solution was freeze-dried to give a white powder (11 mg) of WF11899A.

The fractions containing WF11899B and C on the above-mentioned CPC step were concentrated *in vacuo* and separated on a preparative HPLC column of LiChrosorb RP-18 (E. Merck, 7 $\mu$m, 250 × 25 mm i.d.) using a mobile phase of 45% aqueous $CH_3CN$ containing 0.5% $NH_4H_2PO_4$. The fractions containing one of the two components were combined, diluted with an equal volume of water and passed through a column (1 ml) of ODS-AM. The column was washed with 40% aqueous MeOH and eluted with MeOH. The eluate was concentrated to dryness, then dissolved in water and freeze-dried to give WF11899B (2.2 mg) and WF11899C (1.2 mg) as a white powder, respectively.

## Physico-chemical Properties

The physico-chemical properties of WF11899A, B and C are summarized in Table 2. WF11899A is soluble in MeOH and water, and insoluble in $CHCl_3$. WF11899B and C are soluble in MeOH, slightly soluble in water and insoluble in $CHCl_3$. These compounds give positive color reactions to ceric sulfate and iodine vapor, and negative to Dragendorff and Ehrlich reagents. The UV absorption patterns of them are quite similar to those of the echinocandin-type lipopeptides. Molecular weights of WF11899A, B and C were determined to be 1174, 1158 and 1142, respectively, on the basis of FAB-MS data. Fragment ions $(M+Na-SO_3)^+$ in FAB-MS of WF11899A, B and C were observed at *m/z* 1117, *m/z* 1101 and *m/z* 1085, respectively, suggesting the presence of sulfate group. This is confirmed by enzymatic desulfation of WF11899A by arylsulfatase. The $^1H$ NMR and $^{13}C$ NMR spectra of WF11899A, B and C are shown in Figs. 4 and 5, respectively. The details of the structural elucidation will be described elsewhere.

## Biological Properties

WF11899A, B and C possess similar anti-fungal spectra to the echinocandin family which was

Table 2. Physico-chemical properties of WF11899A, B and C.

| | WF11899A | WF11899B | WF11899C |
|---|---|---|---|
| MP | 215~221°C (dec) | 218~223°C (dec) | 208~217°C (dec) |
| $[\alpha]_D^{23}$ | −20.3° (*c* 0.5, $H_2O$) | −10.5° (*c* 0.5, MeOH) | −9.4° (*c* 0.5, MeOH) |
| Molecular formula for free acid | $C_{51}H_{82}N_8O_{21}S$ | $C_{51}H_{82}N_8O_{20}S$ | $C_{51}H_{82}N_8O_{19}S$ |
| FAB-MS (*m/z*) | | | |
| $(M+2Na-H)^+$ | 1219 | 1203 | 1187 |
| $(M+Na-SO_3)^+$ | 1117 | 1101 | 1085 |
| HRFAB-MS (*m/z*) | | | |
| Obsd: | 1219.5078 | 1203.5100 | 1187.5139 |
| Calcd $(M+2Na-H)^+$: | 1219.5032 | 1203.5083 | 1187.5134 |
| UV $\lambda_{max}^{MeOH}$ nm ($\varepsilon$) | 207 (20,000), 225 (sh), 276 (1,600), 283 (sh) | 206 (23,000), 243 (sh), 278 (460), 284 (sh) | 205 (21,000), 224 (sh), 276 (1,500), 283 (sh) |
| $\lambda_{max}^{MeOH+0.01\,N\,NaOH}$ | 209 (27,000), 244 (7,000), 284 (1,600), 294 (sh) | 208 (29,000), 241 (sh), 290 (600) | 208 (30,000), 241 (sh), 281 (1,400), 295 (sh) |
| IR $\nu_{max}$ (KBr) $cm^{-1}$ | 3350, 2920, 2840, 1660, 1625, 1530, 1510, 1435, 1270, 1240, 1070, 1045, 800, 755, 710 | 3300, 2900, 2840, 1680, 1660, 1640, 1620, 1510, 1460, 1430, 1330, 1240, 1040, 960 | 3350, 2900, 2840, 1680, 1660, 1640, 1620, 1510, 1430, 1330, 1245, 1080, 1040, 960 |
| TLC[a] (Rf value) | 0.31 | 0.67 | 0.90 |

[a] Silica gel 60 $F_{254}$ plate (E. Merck); EtOAc-*i*-PrOH-$H_2O$ (5:3:1).

Fig. 4. $^1H$ NMR spectra of WF11899A, B and C (400 MHz, $CD_3OD$).
(A) WF11899A, (B) WF11899B, (C) WF11899C.

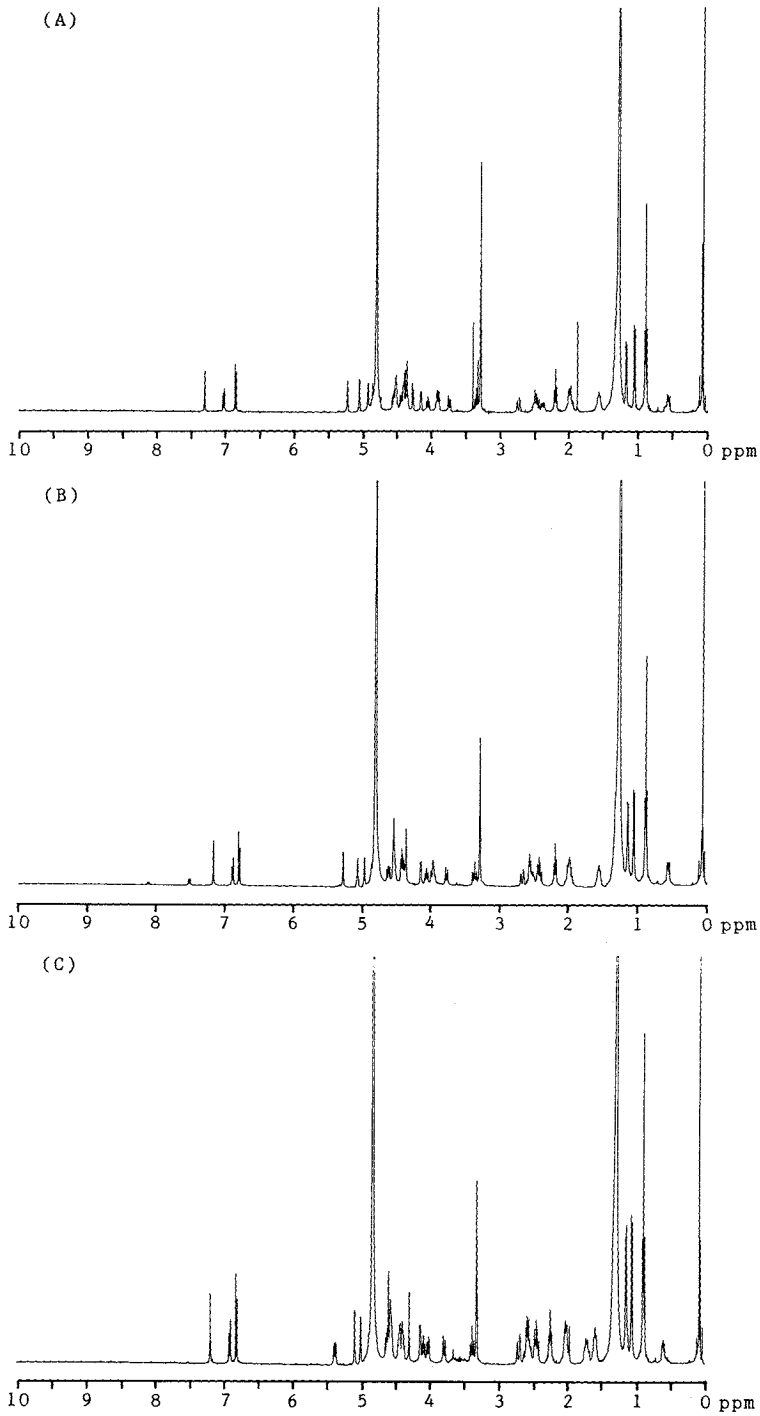
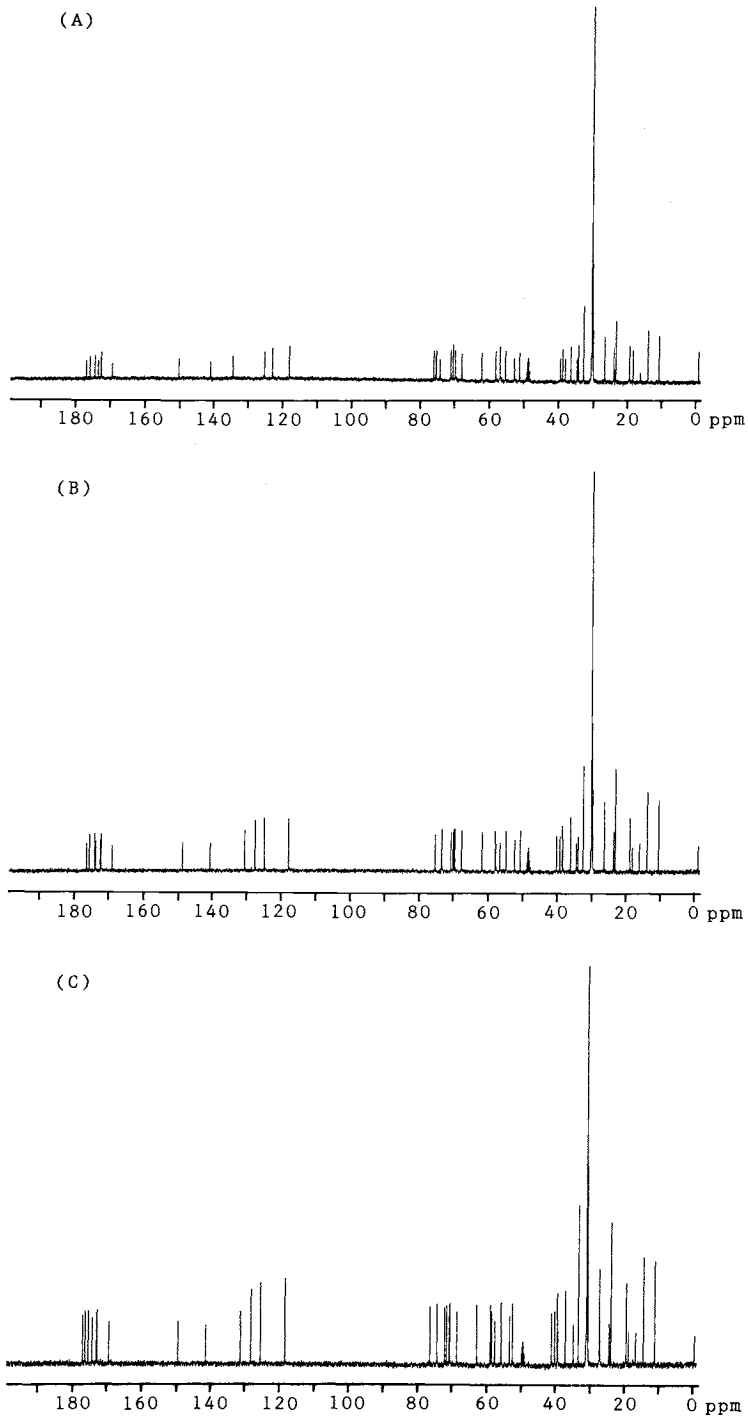


Fig. 5. $^{13}C$ NMR spectra of WF11899A, B and C (100 MHz, $CD_3OD$).
(A) WF11899A, (B) WF11899B, (C) WF11899C.



characterized by excellent activity against *Candida* sp., but little activity against *Cryptococcus* sp. The acute toxicity of WF11899A was determined with an intraperitoneal injection to ICR mice (female, 4 weeks old). No lethal toxicity was observed at the dose of 500 mg/kg. The studies of *in vitro* and *in vivo* antifungal activities of these compounds will be reported in the following paper[13].

## Discussion

A number of echinocandin analogs, which differ in amino acid of the cyclic hexapeptide and fatty acid chain, have been isolated from fungal origin. However, to the best of our knowledge, no analog having a sulfate moiety has been known. Our compounds were reported for the first time to be analogs with a sulfate moiety.

The poor water-solubility has been an impediment to development of echinocandin type of antibiotics. Merck's researchers recently reported chemical modifications[14,15] of natural pneumocandin $B_0$ to overcome this obstacle. We newly discovered water-soluble echinocandin analogs, WF11899A, B and C from a fungal broth. In particular, WF11899A was easily soluble in water even at the concentration of 50 mg/ml. The observation, that the treatment of WF11899A with arylsulfatase diminished its water-solubility, suggested that its excellent solubility should be attributed to the sulfate moiety in its structure.

## Experimental

### General

Melting points were taken on a Yanagimoto micro melting point apparatus and uncorrected. IR spectra were measured on a Shimazu IR-420 3S-48 spectrometer. Optical rotation was determined on a Jasco DIP-140 polarimeter, using 10 cm-microcell. $^1$H NMR spectra (400 MHz) and $^{13}$C NMR (100 MHz) spectra were measured with a Bruker AM400wb spectrometer controlled under an ASPECT 3000 computer. Low-resolution and high-resolution FAB-MS spectra were obtained on a VG ZAB-SE mass spectrometer. Centrifugal partition chromatography was carried on a MODEL-NMF made by Sanki engineering Ltd., Kyoto, Japan.

### Antifungal Activity

Biological activity was assayed through the fermentation and the isolation by a conventional paper disc diffusion assay on agar plate using minimum essential medium (MEM) against *Candida albicans*-7, a strain of Fujisawa culture collection.

### HPLC Analysis

The quantitative and qualitative analysis of WF11899A, B and C were carried out by reverse phase HPLC (LiChrospher 100 RP-18, 5 $\mu$m, 250 × 4 mm i.d., E. Merck) using a solvent system of 45% aqueous $CH_3CN$ containing 0.5% $NH_4H_2PO_4$ (UV detection; 210 nm and flow rate; 1 ml/minute). The retention times of WF11899A, B and C were 11.3, 13.4 and 15.7 minutes, respectively.

### Enzymatic Desulfation of WF11899A

To a solution of WF11899A (60 mg) in 50 mM Tris-HCl buffer (pH 7.1, 30 ml) was added sulfatase (Arylsulfatase; E.C. 3.1.6.1) Type VI from *Aerobacter aerogenes* (Sigma, No. S-1629). After incubating at 37°C for 30 hours, the resultant desulfated WF11899A was extracted with an equal volume of *n*-BuOH. The extract was concentrated to dryness, applied to a column of LiChroprep RP-18 (40~63 $\mu$m) pre-packed size B (25 × 310 mm) equilibrated with 47% aqueous $CH_3CN$ containing 0.5% $NH_4H_2PO_4$, and developed with the same solvent. The active fractions were diluted with an equal volume of water and passed through a column (5 ml) of ODS-AM. The column was washed with water and eluted with MeOH. The eluate was evaporated *in vacuo* to remove MeOH and freeze-dried to give a white powder of desulfated WF11899A (26 mg) having the following properties: HRFAB-MS *m/z* 1117.5659 (Calcd. for $C_{51}H_{82}N_8O_{18}$ Na: 1117.5645); $[\alpha]_D^{22}$ −30° (*c* 1.0, MeOH); UV $\lambda_{max}^{MeOH}$ nm ($\varepsilon$): 207 (39,000), 232 (sh), 282

(2,700); $\lambda_{max}^{MeOH+0.01\,N\,NaOH}$ nm (ε): 208 (51,000), 246 (6,000), 293 (3,400); solubility: soluble in MeOH, slightly soluble in water and EtOAc, insoluble in $CHCl_3$; HPLC retention time 14.7 minutes under the above-mentioned condition (see HPLC analysis in Experimental).

Acknowledgments

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