

## ORIGINAL ARTICLE

# Saprolmycins A–E, new angucycline antibiotics active against *Saprolegnia parasitica*

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Saprolmycins A–E, five new anti-*Saprolegnia parasitica* antibiotics were isolated from the culture broth of *Streptomyces* sp. strain TK08046. As determined using a combination of NMR and spectroscopic analyses, the structures of these compounds were elucidated as a new group of angucycline compounds closely related to saquayamycin. Saprolmycin A and E exhibited potent anti-*S. parasitica* selective activities, with MIC values of 3.9 and 7.8 ng ml<sup>-1</sup>, respectively, but weak or no activity against fungi, gram-positive or -negative bacteria, and microalgae or zooplankton. Our results suggest these two compounds as highly effective and environmentally safe anti-saprolegniasis candidates.

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## INTRODUCTION

Oomycetes represent a diverse group of plant and animal pathogens; *Phytophthora infestans* is one of the most important potato and tomato pathogens, *Pythium insidiosum* is an oomycete that can cause serious life-threatening infections in humans and animals, and the water-mold family Saprolegniaceae including *Saprolegnia parasitica* are fish and crayfish pathogens in ecosystems and can cause enormous damage to fish farms.<sup>1</sup> The lifecycle of Saprolegniaceae includes stages similar to those of the true fungi, such as hyphal elongation, spore formation and germination.<sup>2</sup> Important distinction of Saprolegniaceae includes the presence of cellulose and chitin in their cell walls (true fungi contain only chitin) and their relatedness to brown algae, as determined by 18S rDNA analysis.<sup>3–5</sup> In the aquaculture industry, saprolegniasis has been controlled effectively with malachite green, although recent studies have shown this compound to be environmentally toxic.<sup>6–12</sup> Research conducted over the past two decades has reported mutagenic, teratogenic and residual unfavorable activities of malachite green leading to its ban in many countries.<sup>7–12</sup> A broad-spectrum bactericide substance known as bronopol has been used for preventing saprolegniasis outbreak in salmon and trout eggs populations. However, due to its broad bioactivity<sup>13</sup> and demonstrated toxicities against environmental zooplankton and phytoplankton, bronopol products must be heavily diluted before being discarded, thus increasing usage costs significantly. New and effective anti-saprolegniasis drugs are urgently needed.

During the course of our screening for selective anti-*S. parasitica* compounds from actinomycete cultural broths, we discovered five new angucycline-type antibiotics, namely saprolmycins A (1), B (2), C

(3), D (4) and E (5). In our study, these five compounds all exhibited selective activities, with A (1) and E (5) exhibiting exceptional potency and selectivity as potential anti-*S. parasitica* compounds. We report herein the isolation, structure elucidation and biological effects of saprolmycins.

## MATERIALS AND METHODS

### General experimental procedures

All reagents in our experiments were purchased from Wako Pure Chemical Industries, Osaka, Japan. Optical rotations were measured with a DIP-1000 digital polarimeter (JASCO, Tokyo, Japan). NMR spectra were measured using a JNM-ECA-600 NMR spectrometer (JEOL, Tokyo, Japan) with tetramethylsilane as an internal standard in CDCl<sub>3</sub> or CD<sub>3</sub>OD. HPLC analysis was carried out using a HPLC pump GL-7410 and a PDA detector GL-7452 (GL Science, Tokyo, Japan). For HPLC fractionations, a HPLC pump L-6200 (Hitachi, Tokyo, Japan) and a UV detector L-4200 (Hitachi) were used. IR spectra were recorded on a FT/IR-8600 spectrometer (Shimadzu, Kyoto, Japan). HR-FAB-MS spectra were measured on a JMS-700T tandem mass spectrometer (JEOL). ESI-MS spectra were measured with an API-3200 triple quadrupole mass spectrometer (Applied Biosystems, Tokyo, Japan). UV-vis spectra were measured with a V-630 spectrophotometer (JASCO). Optical densities were measured with a microplate reader SH-1000 Lab (Corona, Hitachinaka, Japan). Melting points were measured with MP-J3 (Yanaco, Kyoto, Japan).

### Organisms

The strain TK08046 was isolated from a soil sample collected at Shizuoka City, Japan, which we classified as *Streptomyces* sp. using 16S rDNA sequence analysis with 1516 bp of the genome (gene accession no. AB67946). The primers 10F (5'-GTTTGATCCTGGCTCA-3'), 686F (5'-TAGCGGTGAAATGC GTAGA-3'), 800R (5'-TACCAGGGTATCTAATCC-3'), 1541R (5'-AAGGAGGT GATCCAGCC-3') were used for amplification of the almost complete 16S

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ribosomal DNA gene. TK08046 was deposited in the culture collection of NITE Biological Resource Center (Chiba, Japan) as NITE P-1138. For maintenance, the strain was grown on starch casein agar slant (1.0% starch, 0.03% casein, 0.2% NaCl, 0.2% K<sub>2</sub>HPO<sub>4</sub>, 0.005% MgSO<sub>4</sub>, 0.002% CaCO<sub>3</sub>, 0.001% FeSO<sub>4</sub>, pH 7.2) containing 1.5% of agar.

### Fermentation

A loop of spores of the strain were inoculated into starch casein medium (10 ml) in a test tube, and incubated for 5 days at 30 °C on a reciprocal shaker (120 r.p.m.). The seed-culture was transferred into 200 ml starch casein medium in a Sakaguchi flask (60 flasks, total 121) and incubated for 7 days at 30 °C on a reciprocal shaker (120 r.p.m.).

### Isolation

The cultured broth (121) was filtered and the filtrate was extracted with EtOAc (121). The organic layer was concentrated to dryness and 717.9 mg of the residue was obtained. The extract was dissolved in MeOH and the solution was applied to an octadecylsilane (ODS) column (Wakogel 50C<sub>18</sub>, i.d. 30 × 150 mm). It was eluted with a stepwise gradient of H<sub>2</sub>O–CH<sub>3</sub>CN (30, 50, 70, 90 and 100%) and fractionated. Active fractions of 50–90% CH<sub>3</sub>CN eluate were combined and concentrated *in vacuo* to yield a crude material (329.2 mg). Further purification was carried out using a HPLC ODS column (Cosmosil 5C<sub>18</sub>-AR-II, i.d. 10 × 250 mm, detection 230 nm, flow rate 2.5 ml min<sup>-1</sup>) with 60% CH<sub>3</sub>CN isocratic elution. The effluent was divided into several fractions and each fraction was bioassayed. Five peak fractions covering the beginning to the end of each peak were active and were evaporated under reduced pressure to give the five purified active compounds, saprolmycin A (1: retention time (r.t.) = 8.96 min, 3.1 mg), B (2: r.t. = 11.27 min, 5.9 mg), C (3: r.t. = 11.96 min, 10.6 mg), D (4: r.t. = 15.99 min, 14.9 mg) and E (5: r.t. = 16.96 min, 60.1 mg).

### Complete and partial hydrolysis of 5

Antibiotic 5 was degraded completely<sup>14</sup> or partially<sup>15</sup> by using acid hydrolysis. 5 (30.3 mg) was completely hydrolyzed with 0.5 M H<sub>2</sub>SO<sub>4</sub> for 1 h at 80 °C. An aglycon (7: 7.0 mg) was obtained from the reaction mixture by EtOAc extraction and further purification on a HPLC ODS column (Cosmosil 5C<sub>18</sub>-AR-II, i.d. 10 × 250 mm, detection 230 nm, flow rate 2.5 ml min<sup>-1</sup>) with 20% CH<sub>3</sub>CN isocratic elution.

Antibiotic 5 (20.2 mg) was partially hydrolyzed with CH<sub>3</sub>CN–0.2% phosphoric acid (60:40) for 1 h at 40 °C, and extracted with CHCl<sub>3</sub>. The organic solvent layer was dried *in vacuo* and it was purified on a HPLC ODS column (Cosmosil 5C<sub>18</sub>-AR-II, i.d. 10 × 250 mm, detection 230 nm, flow rate 2.5 ml min<sup>-1</sup>) using 30% CH<sub>3</sub>CN isocratic elution to yield a partial hydrolysate (8: 12.2 mg).

### Measurement of biological activities

*S. parasitica* was cultured on glucose yeast extract agar medium (0.25% yeast extract, 0.25% glucose, 1.5% agar, pH 6.0) for 7 days at 16 °C. A spore suspension was prepared by using a modified method reported by Heath and Greenwood.<sup>16</sup> The spore suspension (10<sup>3</sup> spores ml<sup>-1</sup>, 100 μl), quadruple concentration of glucose yeast extract medium (50 μl) and a serial double diluted sample solution in 8% MeOH–H<sub>2</sub>O (50 μl) were poured into wells of 96-well plate and incubated for 24 h at 16 °C. A solution of malachite green (0.25 μg ml<sup>-1</sup>) was used as a positive control. The growth of vegetative hyphae was observed microscopically (Olympus, CK2) and the MIC of the sample was determined.

Anti-bacterial activities against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas putida* and *Staphylococcus aureus* were determined using the conventional paper disc method with Luria-Bertani agar medium inoculated with each bacterium. Anti-yeast and anti-fungal activities were measured using a method similar to that for the anti-*S. parasitica* assay using a cell suspension of *Saccharomyces cerevisiae* (2.0 × 10<sup>4</sup> cells ml<sup>-1</sup>) and a mycelial suspension of *Phoma* sp. in distilled water. *Phoma* sp. was cultured in Sabouraud agar medium, a piece of the agar medium (1 cm<sup>2</sup>) containing its vegetative hypha was crushed with distilled water to make a mycelial suspension. The *S. cerevisiae* suspension (100 μl) and quadruple concentration of the yeast extract peptone D-glucose medium (4% yeast extract, 8% peptone, 8% glucose, pH 6.5, 50 μl), *Phoma* sp. suspension (100 μl) and Sabouraud medium (50 μl) were mixed separately with a serial

double diluted sample solution in 8% MeOH–H<sub>2</sub>O (50 μl) in wells of 96-well plate and incubated for 24 h at 30 °C. A solution of cycloheximide (25 μg ml<sup>-1</sup>) was used as a positive control. Anti-*S. cerevisiae* and *Phoma* sp. activities were observed microscopically, and the MICs of the samples were determined.

*Synechocystis* sp. and *Chlorella kessleri* were cultured in BG-11 medium and CA medium, respectively, under a light intensity of 25 μmol photons m<sup>-2</sup> s<sup>-1</sup> with a 16:8 h light:dark cycle at 25 °C. Algal cells in logarithmic growth phase were used for the assay. *Synechocystis* sp. suspension (100 μl) and BG-11 medium (50 μl) and *C. kessleri* suspension (100 μl) and CA medium (50 μl) were mixed separately with a serial double diluted sample solution in 8% MeOH–H<sub>2</sub>O (50 μl) in wells of 96-well plate and incubated for 7 days at 25 °C under the same light conditions. A solution of cycloheximide (25 μg ml<sup>-1</sup>) was used as a positive control. Anti-algal activities were observed microscopically and the algal growth was monitored using OD methods, and was determined to be 680 nm with a microplate reader.

The water flea, *Daphnia pulex*, was isolated from a pond in Ritsumeikan University. *D. pulex* was cultured using *C. kessleri* as a food source in clean water under a light intensity of 10 μmol photons m<sup>-2</sup> s<sup>-1</sup> with a 16:8 h light:dark cycle at 18–20 °C. Five offspring born within 24 h were added to a serial 10-fold diluted sample solution (final 1% MeOH, 10 ml) and incubated for 24 h using the above-mentioned conditions. Mortalities were counted, and the value of LD<sub>50</sub> was estimated using a probit model and the statistic software, SPSS (IBM, Tokyo, Japan).

## RESULTS AND DISCUSSION

### Taxonomy of the producing strain TK08046

Strain TK08046 was isolated from a soil sample collected in Shizuoka City, Japan. The vegetative mycelia developed well on starch casein, Bennet, glucose yeast extract and Luria-Bertani agar plates. The color of vegetative mycelia was dark orange and the color of mature spore was white on starch casein agar. On the other agar plates, the mycelia and the spore showed pale dark orange and white, respectively. 16S rDNA region were amplified and sequenced by using representative primers. Almost complete 16S rDNA gene (1516 bp) was sequenced and used for the identification. Comparison of the sequence with known sequences of *Streptomyces* sp. in GeneBank database was carried out by the BLAST search. The BLAST result showed that the 16S rDNA sequence of the strain had 99.5 and 99.3% sequence similarities with *Streptomyces roseogriseus* NBRC13681 and *Streptomyces canaries* NBRC13431, respectively. Therefore, the strain TK08046 was classified as a *Streptomyces* species.

### Isolation and characterization

The fermentation was performed in starch casein medium with aeration, and the filtered culture broth (121) was extracted with EtOAc (121) and the organic layer was evaporated to dryness (717.9 mg). The acquired crude extract was applied to an ODS column and the fractions were eluted with a stepwise gradient of H<sub>2</sub>O–CH<sub>3</sub>CN. The fractions of 50–90% CH<sub>3</sub>CN eluate were combined and concentrated *in vacuo* to yield a crude material (329.2 mg). Further purification was carried out with a HPLC ODS column to obtain five active compounds, saprolmycin A (1: r.t. = 8.96 min, 3.1 mg), B (2: r.t. = 11.27 min, 5.9 mg), C (3: r.t. = 11.96 min, 10.6 mg), D (4: r.t. = 15.99 min, 14.9 mg) and E (5: r.t. = 16.96 min, 60.1 mg). All compounds were dark-yellow powder and soluble in MeOH, EtOAc and CHCl<sub>3</sub>, but insoluble in water and *n*-hexane. The UV-vis and IR data of these compounds closely resembled each other, which indicated they were analogs containing the same chromophore. The physicochemical properties of these compounds are listed in Table 1.

### Structure determination

*Saprolmycin E* (5). Comprehensive analyses of 2D NMR spectra (COSY, HMQC, HMBC and ROESY) conducted on the compounds

**Table 1** Physicochemical properties of saprolmycin A (1)–E (5), aglycon (7) and partial hydrolysate (8)

	1	2	3	4	5	7	8
Appearance	Dark yellow powder	Dark yellow powder	Dark yellow powder	Dark yellow powder	Dark yellow powder	Dark yellow powder	Dark yellow powder
ESI-MS ( <i>m/z</i> )	705 [M-H] <sup>-</sup>	707 [M-H] <sup>-</sup>	705 [M-H] <sup>-</sup>	821 [M-H] <sup>-</sup>	819 [M-H] <sup>-</sup>	485 [M-H] <sup>-</sup>	595 [M-H] <sup>-</sup>
HR-FAB-MS ( <i>m/z</i> )							
Found	713.2407 [M + Li] <sup>+</sup>	715.2603 [M + Li] <sup>+</sup>	713.2407 [M + Li] <sup>+</sup>	829.3266 [M + Li] <sup>+</sup>	827.3089 [M + Li] <sup>+</sup>	485.1451 [M-H] <sup>-</sup>	595.1811 [M-H] <sup>-</sup>
Calculated	713.2422 [M + Li] <sup>+</sup>	715.2578 [M + Li] <sup>+</sup>	713.2422 [M + Li] <sup>+</sup>	829.3259 [M + Li] <sup>+</sup>	827.3102 [M + Li] <sup>+</sup>	485.1448 [M-H] <sup>-</sup>	595.1815 [M-H] <sup>-</sup>
Molecular formula	C <sub>37</sub> H <sub>38</sub> O <sub>14</sub>	C <sub>37</sub> H <sub>40</sub> O <sub>14</sub>	C <sub>37</sub> H <sub>38</sub> O <sub>14</sub>	C <sub>43</sub> H <sub>50</sub> O <sub>16</sub>	C <sub>43</sub> H <sub>48</sub> O <sub>16</sub>	C <sub>25</sub> H <sub>26</sub> O <sub>10</sub>	C <sub>31</sub> H <sub>32</sub> O <sub>12</sub>
UV λ <sub>max</sub>	217 (4.65), 317	218 (4.62), 315	218 (4.65), 316	219 (4.68), 316	217 (4.67), 318	219 (4.36), 319	219 (4.78), 320
(MeOH) nm	(3.85), 425 (3.78)	(3.85), 423 (3.89)	(3.86), 425 (3.79)	(3.91), 429 (3.88)	(3.88), 427 (3.90)	(3.77), 429 (3.69)	(3.96), 432 (3.99)
(log $\epsilon$ )							
IR ν <sub>max</sub>	1732, 1699, 1639	1732, 1701, 1639	1734, 1701, 1643	1732, 1701, 1641	1730, 1701, 1639	1718, 1683, 1645	1718, 1684, 1645
(film) cm <sup>-1</sup>							
m.p. (°C)	174–179	164–167	167–170	170–172	175–177	180–181	180–182
[α] <sub>D</sub> <sup>25</sup>	+79 (c 0.1, CHCl <sub>3</sub> )	-131 (c 0.1, CHCl <sub>3</sub> )	-124 (c 0.1, CHCl <sub>3</sub> )	-59 (c 0.1, CHCl <sub>3</sub> )	+76 (c 0.1, CHCl <sub>3</sub> )	+152 (c 0.1, dioxane) +184 (c 0.14, MeOH)	+42 (c 0.25, CHCl <sub>3</sub> )

revealed significant similarities and differences discussed herein. The structural elucidation of a major component **5** was carried out at first. The molecular formula of **5** was elucidated to be C<sub>43</sub>H<sub>48</sub>O<sub>16</sub> from HR-FAB-MS data (found *m/z* 827.3089 [M + Li]<sup>+</sup>, calcd. 827.3102 [M + Li]<sup>+</sup>). The molecular formula coincided with that of saquayamycin A (**6**), an angucycline antibiotic that consists of three sugar moieties, a rhodinoside and two aculosides, and an aglycon containing benz[a]anthraquinone chromophore and a characteristic saturated pyran ring. All spectroscopic data (IR, UV-vis, and <sup>1</sup>H NMR) of **5** were also similar to those of **6**.<sup>15</sup> In the <sup>13</sup>C NMR spectrum of **5**, many signals were observed with <1 p.p.m. variation from published data of **6**; however, chemical shifts of four signals at around 90–100 p.p.m. seemed to be somewhat different. By detailed comparison of the data, chemical shifts of two carbon signals at δ<sub>C</sub> 88.4 and 98.9 p.p.m. in **5** were considered to be observed with a difference of 4.0 and 3.6 p.p.m., respectively, from reported chemical shifts (δ<sub>C</sub> 92.4 and 95.3 p.p.m.) in **6**. These two carbons in **6** were attributed to anomeric carbons.

The <sup>1</sup>H NMR spectrum of **5** suggested the existence of the same aglycon in **6**, that is, a hydrogen bonding phenolic hydroxyl group 8-OH (δ<sub>H</sub> 12.30); three exchangeable hydroxy groups, 4a-OH (δ<sub>H</sub> 3.69), 12b-OH (δ<sub>H</sub> 4.57) and 3'-OH (δ<sub>H</sub> 4.93); and four aromatic protons, H-5 (δ<sub>H</sub> 6.39), H-6 (δ<sub>H</sub> 6.89), H-11 (δ<sub>H</sub> 7.59) and H-10 (δ<sub>H</sub> 7.86). To confirm the structure and the absolute configuration of the aglycon, **5** was hydrolyzed with 0.5 M H<sub>2</sub>SO<sub>4</sub> for 1 h at 80 °C and an aglycon (**7**) was obtained. The physicochemical properties and NMR data of **7** were listed in Tables 1 and 2, respectively. **7** was identified to be the aglycon of **6** including stereochemistry by comparison with published <sup>1</sup>H NMR<sup>14</sup> and optical rotation data (lit.,<sup>15</sup> [α]<sub>D</sub><sup>28</sup> +151, c 0.2, dioxane). The structure of **7** was displayed in Figure 3. <sup>1</sup>H and <sup>13</sup>C NMR signals of the aglycon in **5** were distinguished by COSY and HMBC data summarized in Figure 1.

Remaining NMR signals of **5** were three methyl, two methylene, four oxygenated methine, three anomeric and four olefinic methine proton and carbon signals, and two ketone carbon ones. COSY and HMBC data of **5** revealed three sugar moieties of two kinds of 2, 3, 6-trideoxyhexoses. An existence of a rhodinoside or an amicitoside, of which 4-hydroxyl and 6-methyl groups were *cis* or *trans*

configuration, respectively, was indicated by tracing COSY correlations summarized in Figure 1. A rhodinoside was strongly suggested by a small coupling constant (*J* = 1.5 Hz) between H''-4 and H''-5 protons, because large value as axial-axial coupling was expected and indeed *J* = 8.5 Hz was observed in an amicitoside in landomycin.<sup>17</sup> Two aculosides containing α, β-unsaturated ketone were uncovered by the combination of COSY and HMBC data summarized in Figure 1. Thus, four components, an aglycon, a rhodinoside and two aculoside moieties were disclosed and these partial resolved structures of **5** were identical to the components of **6**.

The difference between **5** and **6** is the linkage order of sugars and the aglycon. The central HMBC correlations establishing connections among the three sugars and the aglycon were follows; the rhodinoside connected to C-4'-OH of the aglycon pyran (H-1'' to C-4'), an aculoside connected to C-4''-OH of the rhodinoside (H-1''' to C-4''), and a second aculoside connected to C-3-OH of the aglycon chromophore (H-1'''' to C-3), that is, a disaccharide (aculosylrhodinoside) and an aculoside connected to the aglycon pyran and chromophore, respectively. These connections were also supported by ROESY correlation data as shown in Figure 2, thus the structure of **5** was determined as shown in Figure 3. In the case of **6**, a disaccharide (aculosylrhodinoside) and an aculoside connected to opposite positions of the aglycon, and the differences of chemical shifts of anomeric carbons in **5** and **6** should be caused by these structural differences.

In order to confirm the structure, **5** was partially hydrolyzed with CH<sub>3</sub>CN–0.2% phosphoric acid for 1 h at 40 °C to yield a partial hydrolysate (**8**). The physicochemical properties as shown Table 1 and <sup>1</sup>H NMR data of **8** suggested the lack of the disaccharide aculosylrhodinoside moiety from **5**. The aglycon pyran C-4' signal was observed at δ<sub>C</sub> 77.5 in the <sup>13</sup>C NMR spectrum of **8**, which was higher than that (δ<sub>C</sub> 88.4) in **5**, also indicated the lack of the disaccharide. 2D NMR analyses of **8** summarized in Figure 4 demonstrated the connection between an aculoside to C-3 of the aglycon chromophore *via* a glycosidic bond. The structure of **5** was established by that of **8** because an aculoside could connect to only one moiety *via* a glycosidic bond and thus should be the non-reducing end of the disaccharide in **5**.

**Table 2** Assignments of  $^1\text{H}$  and  $^{13}\text{C}$  NMR signals of saprolmycin E (5), saquayamycin A (6), aglycon (7) and partial hydrolysate (8)

Position	5 <sup>a</sup>		6 <sup>b</sup>		7 <sup>c</sup>		8 <sup>c</sup>	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult, J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult, J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult, J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult, J in Hz)
1	203.6		204.7		208.0		205.8	
2	49.7	2.53 (d, 13.0)	50.2	2.51 (d)	54.3	2.65 (d, 13.1)	50.6	2.73 (d, 13.1)
		3.20 (dd, 3.4, 13.0)		3.18 (dd)		2.82 (d, 3.4, 13.1)		2.90 (dd, 3.4, 13.1)
3	82.3		82.4		78.7		82.6	
4	42.1	1.80 (d, 15.1)	44.5	1.85 (d)	45.7	1.99 (d, 15.0)	41.4	1.94 (d, 15.8)
		2.44 (dd, 3.4, 15.1)		2.29 (dd)		2.03 (d, 15.0)		2.43 (dd, 2.8, 15.8)
4a	78.8		79.9		79.7		79.4	
4a-OH		3.69 (brs)						
5	144.8	6.39 (d, 9.6)	145.6	6.46 (d)	147.3	6.37 (d, 9.6)	146.1	6.38 (d, 9.6)
6	116.9	6.89 (d, 9.6)	117.4	6.91 (d)	119.2	6.84 (d, 9.6)	116.5	6.85 (d, 9.6)
6a	138.0		138.8		141.4		139.0	
7	187.6		188.2		190.8		188.9	
7a	113.4		114.0		116.4		114.6	
8	157.6		158.0		159.8		158.0	
8-OH		12.3 (brs)		12.29 (s)				
9	138.1		138.2		140.3		138.6	
10	133.2	7.86 (d, 7.6)	133.5	7.88	135.6	7.83 (d, 7.6)	133.3	7.84 (d, 7.6)
11	119.3	7.59 (d, 7.6)	119.6	7.61	121.0	7.59 (d, 7.6)	118.9	7.56 (d, 7.6)
11a	129.9		130.5		133.2		131.6	
12	181.8		182.2		184.6		182.9	
12a	138.2		138.9		140.9		139.3	
12b	76.7		77.4		83.1		70.3	
12b-OH		4.57 (brs)						
13	26.0	1.45 (s)	25.5	1.41 (s)	31.2	1.20 (s)	26.3	1.39 (s)
1'	70.5	4.83 (dd, 1.4, 10.3)	71.1	4.89 (dd)	73.5	4.77 (d, 11.0)	71.1	4.85 (m)
2'	38.2	1.36 (dd, 5.2, 13.1)	38.9	1.38–1.48 (m)	42.1	1.34 (m)	38.2	1.30 (m)
		2.49 (ddd, 1.4, 5.2, 13.1)		2.57 (ddd)		2.41 (ddd, 1.4, 5.2, 11.0)		2.40 (ddd, 2.1, 4.8, 13.1)
3'	70.9	3.79 (m)	71.3	3.91 (dddd)	74.7	3.69 (m)	72.4	3.67 (m)
3'-OH		4.93 (brs)		4.26 (d)				
4'	88.4	3.04 (m)	89.3	3.21 (dd)	79.8	3.02 (t, 9.6)	77.5	3.01 (t, 8.9)
5'	74.0	3.54 (m)	74.4	3.58 (dq)	78.8	3.41 (m)	76.4	3.42 (m)
6'	18.0	1.34 (d, 6.2)	18.4	1.39 (d)	19.6	1.35 (d, 6.8)	17.5	1.34 (d, 6.2)
1''	98.9	4.97 (brs)	95.2	5.38 (d, 3.5)			88.5	5.64 (d, 4.1)
2''	23.8	1.68 (m)	142.1	6.85 (dd)			145.3	6.85 (dd, 4.5, 10.3)
		2.10 (m)						
3''	24.6	1.93 (m)	127.3	6.15 (d, 10.2)			126.5	5.97 (d, 10.3)
		2.08 (m)						
4''	75.6	3.69 (brs)	195.1				198.4	
5''	67.3	4.22 (dq, 1.5, 6.8)	71.6	4.76 (q)			70.2	4.68 (q, 6.9)
6''	16.5	1.25 (d, 6.8)	15.2	1.45 (d)			14.5	1.32 (d, 6.9)
1'''	94.8	5.24 (d, 3.4)	92.4	5.26 (brd)				
2'''	142.2	6.86 (dd, 3.4, 10.3)	24.7	1.49 (m)				
				2.04 (m)				
3'''	127.0	6.04 (d, 10.3)	24.5	1.90 (m)				
				1.92 (m)				
4'''	196.1		76.2	3.70 (ddd)				
5'''	70.2	4.55 (q, 6.9)	67.0	4.25 (dq)				
6'''	14.6	1.41 (d, 6.9)	17.1	1.29 (d)				
1''''	88.2	5.57 (d, 4.1)	95.3	5.27 (d, 3.5)				
2''''	142.4	6.67 (dd, 4.1, 10.3)	143.0	6.89 (dd)				
3''''	127.1	6.09 (d, 10.3)	127.3	6.09 (d, 10.2)				
4''''	196.3		196.7					
5''''	70.1	4.71 (q, 6.2)	70.7	4.55 (q)				
6''''	14.7	1.37 (d, 6.2)	15.1	1.37 (d)				

<sup>a</sup>Measured in CDCl<sub>3</sub>.<sup>b</sup>Uchida *et al.*<sup>15</sup><sup>c</sup>Measured in CD<sub>3</sub>OD.

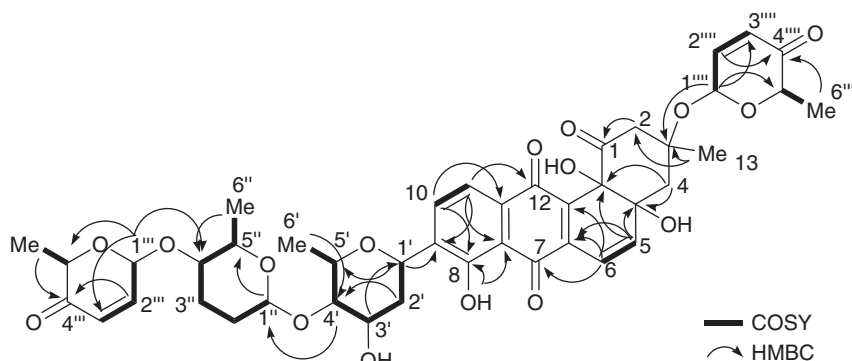


Figure 1 Summarized COSY and HMBC correlations of saprolmycin E (5).

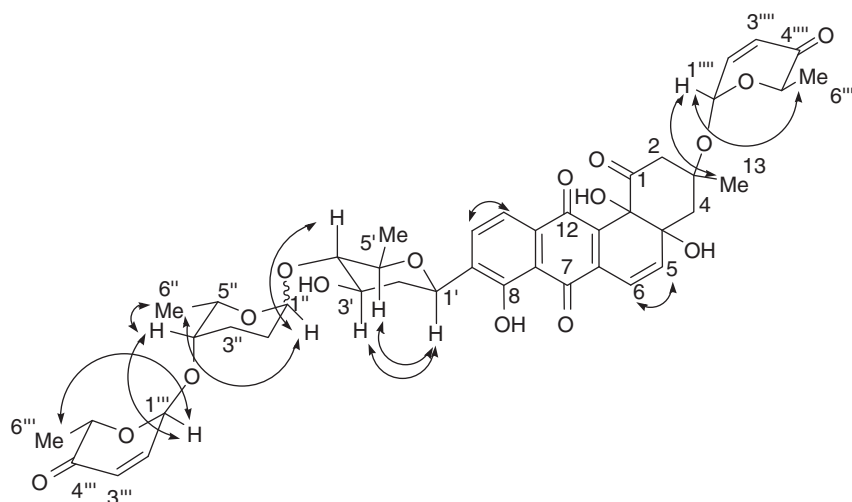


Figure 2 Selected ROESY correlations of saprolmycin E (5).

The glycosidic bonds of two aculoses in **5** should be  $\beta$ -form because  $J$  values of aculose anomeric  $\alpha$ -protons in **6**<sup>15</sup> and P-1894B<sup>14</sup> were reported to be 3.4 Hz and the  $J$  values of those in **5** were 3.4 and 4.1 Hz. The total assignments of  $^1\text{H}$  and  $^{13}\text{C}$  NMR signals of **5** were demonstrated in Table 2. The structures of **1–4** isolated as minor components from the same fermented broth were determined by comparisons of spectral data with those of **5** and each other.

**Saprolmycin D (4)**. The ESI-MS spectrum of **4** showed a pseudo-molecular ion peak  $[\text{M}-\text{H}]^-$  at  $m/z$  821, the UV-vis and IR data of **4** as shown in Table 1 closely resembled those of **5** and **6**.<sup>15</sup> The molecular formula of **4** was determined to be  $\text{C}_{43}\text{H}_{50}\text{O}_{16}$  from its HR-FAB-MS data (found  $m/z$  829.3266  $[\text{M} + \text{Li}]^+$ , calcd.  $m/z$  829.3259  $[\text{M} + \text{Li}]^+$ ), and it revealed that **4** should be a dihydro derivative of **5** from their molecular formulas. The signals of the aglycon, an aculose and a rhodnose were observed in  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra. However, two olefinic proton signals of another aculose in **5** had disappeared and four aliphatic proton signals ( $\text{H}-2''''$  ( $\delta_{\text{H}}$  1.86 and  $\delta_{\text{H}}$  2.29) and  $\text{H}-3''''$  ( $\delta_{\text{H}}$  2.36–2.38, 2H)) were observed in  $^1\text{H}$  NMR spectrum of **4**. The presence of a cinerulose, which was a dihydro derivative of an aculose, was indicated from the combination of COSY and HMBC data. This sugar bonded to C-3 of the aglycon chromophore *via* a glycosidic bond from HMBC data ( $\text{H}-1''''$  ( $\delta_{\text{H}}$  5.40) to C-3 ( $\delta_{\text{C}}$  82.5)) and thus the structure of **4** was determined as shown in Figure 3. The

total assignments of  $^1\text{H}$  and  $^{13}\text{C}$  NMR signals were demonstrated in Table 3.

**Saprolmycin A (1)**. The ESI-MS spectrum of **1** showed a pseudo-molecular ion peak  $[\text{M}-\text{H}]^-$  at  $m/z$  705, the UV-vis and IR data as shown in Table 1 closely resembled those of **5** and **6**.<sup>15</sup> The molecular formula of **1** was elucidated to be  $\text{C}_{37}\text{H}_{38}\text{O}_{14}$  from its HR-FAB-MS data (found  $m/z$  713.2407  $[\text{M} + \text{Li}]^+$ , calcd.  $m/z$  713.2422  $[\text{M} + \text{Li}]^+$ ), which indicated the lack of  $\text{C}_6\text{H}_{10}\text{O}_2$  corresponding to a rhodnose residue from **5**. There were no signals of a rhodnose moiety in the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **1** but those of two aculoses were observed. HMBC correlations from the two aculose anomeric protons to the aglycon carbons ( $\text{H}-1''$  ( $\delta_{\text{H}}$  5.37) to C-4' ( $\delta_{\text{C}}$  89.4) and  $\text{H}-1'''$  ( $\delta_{\text{H}}$  5.57) to C-3 ( $\delta_{\text{C}}$  82.8)) indicated aculoses directly connected on the aglycon moiety at C-3 and C-4' *via* a glycosidic bond. Thus, the structure of **1** was determined as shown in Figure 3 and the assignments of  $^1\text{H}$  and  $^{13}\text{C}$  NMR signals were listed in Table 3. The coupling constants of anomeric signals,  $\text{H}-1''$  ( $J=3.4$  Hz), and  $\text{H}-1'''$  ( $J=3.4$  Hz), indicated that the aculoses in **1** were connected by  $\beta$ -linkage.

**Saprolmycin C (3)**. The ESI-MS spectrum of **3** showed a pseudo-molecular ion peak  $[\text{M}-\text{H}]^-$  at  $m/z$  705, the UV-vis and IR data as shown in Table 1 closely resembled those of **5** and **6**.<sup>15</sup> The molecular

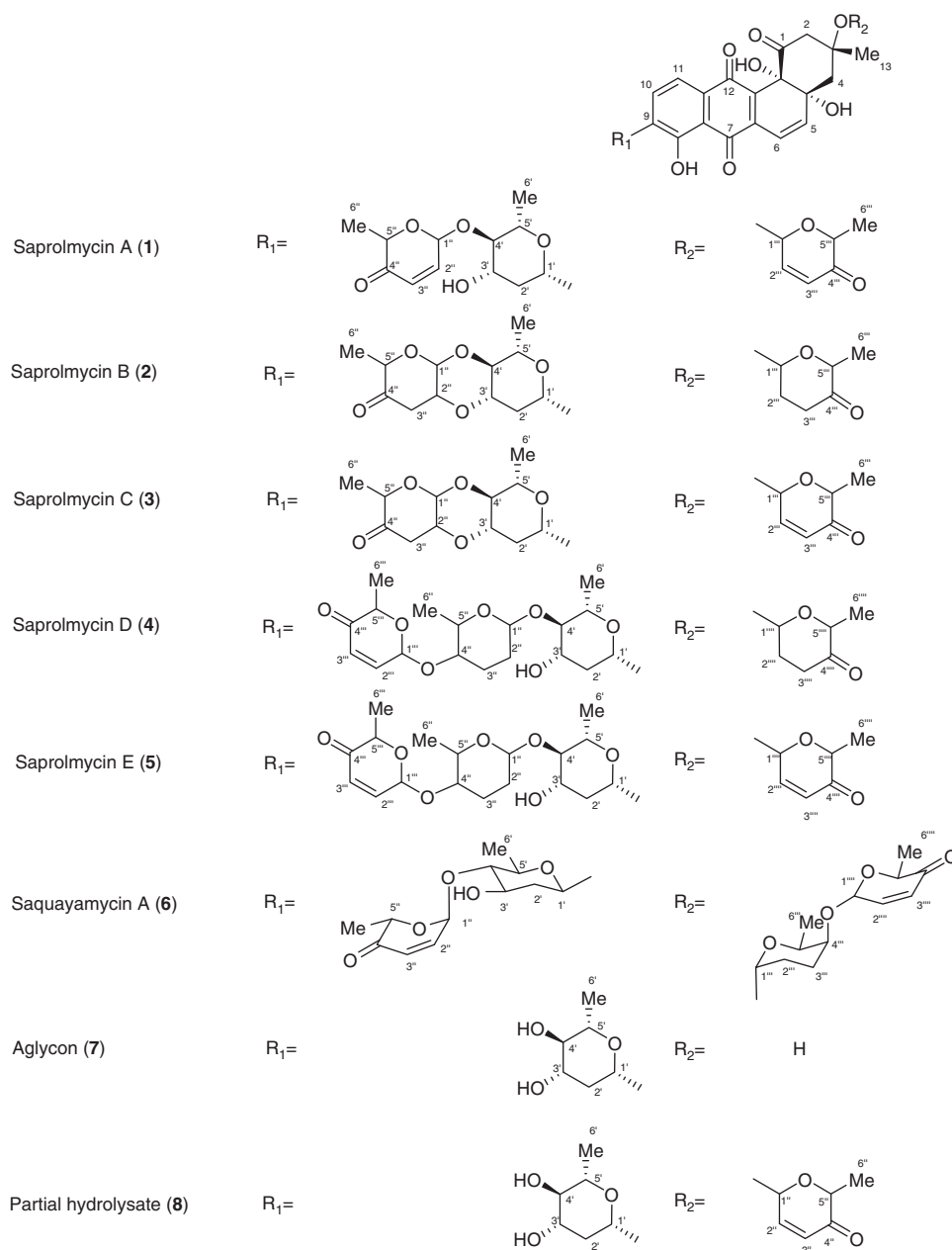


Figure 3 Structures of saprolmycin A (1)–E (5), saquayamycin A (6), aglycon (7) and partial hydrolysate (8).

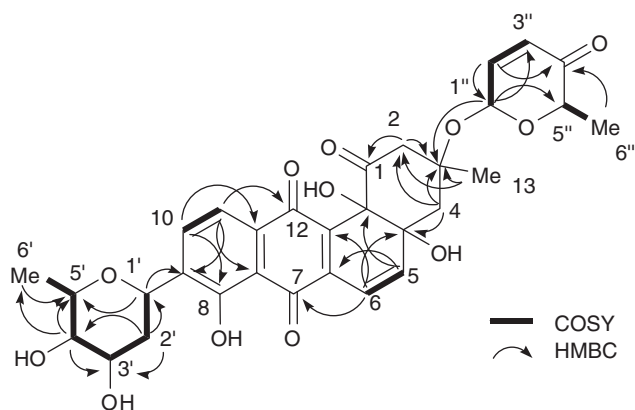


Figure 4 Summarized COSY and HMBC correlations of partial hydrolysate (8).

formula of **3** was elucidated to be C<sub>37</sub>H<sub>38</sub>O<sub>14</sub> from its HR-FAB-MS data (found  $m/z$  713.2407 [M + Li]<sup>+</sup>, calcd.  $m/z$  713.2422 [M + Li]<sup>+</sup>), which was the same molecular formula of **1**. Comparing the NMR spectra of **1** and **3**, we found an aculose which bonded to C-3 of the aglycon chromophore *via* a glycosidic bond from HMBC data (H-1'''' ( $\delta_H$  5.57) to C-3 ( $\delta_C$  43.2)) but did not find another aculose moiety. The olefinic proton of second aculose signals in **1** disappeared in <sup>1</sup>H NMR spectrum of **3** but three aliphatic protons, an oxygenated methine (H-2'' ( $\delta_H$  4.33)) and a methylene (H-3'' ( $\delta_H$  2.62–2.66, 2H)) were observed in **3**. COSY and HMBC data revealed the presence of a 2-oxygenated cinerulose moiety and furthermore HMBC correlation data (H-1'' ( $\delta_H$  5.17) to C-4' ( $\delta_C$  75.1); H-3'' ( $\delta_H$  3.81) to C-2'' ( $\delta_C$  71.8)) indicated the formation of a dioxane ring between the aglycon pyran and the cinerulose moieties. Thus, the structure of **3** was determined as shown

Table 3 Assignments of  $^1\text{H}$  and  $^{13}\text{C}$  NMR signals of saprolmycin A (1)–D (4)

Position	<b>1<sup>a</sup></b>		<b>2<sup>a</sup></b>		<b>3<sup>a</sup></b>		<b>4<sup>a</sup></b>	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult. J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult. J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult. J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult. J in Hz)
1	204.2		204.5		208.4		204.5	
2	50.2	2.55 (m) 3.23 (m)	50.5	2.51 (d, 13.1) 3.28 (dd, 3.4, 13.1)	50.8	2.54 (d, 13.8) 3.21 (dd, 3.4, 13.8)	50.5	2.51 (d, 13.1) 3.20 (dd, 3.0, 13.1)
3	82.8		82.5		83.3		82.5	
4	42.7	1.81 (d, 15.8) 2.45 (dd, 2.6, 15.8)	44.1	1.79 (d, 15.1) 2.35 (dd, 3.4, 15.4)	43.2	1.80 (d, 15.8) 2.45 (dd, 2.8, 15.8)	44.1	1.79 (d, 15.2) 2.35 (dd, 3.0, 15.2)
4a	79.4		79.9		79.9		79.8	
4a-OH		3.62 (brs)		3.96 (brs)		3.56 (brs)		3.95 (brs)
5	145.3	6.40 (d, 9.6)	145.3	6.42 (d, 9.6)	146.0	6.40 (d, 9.6)	145.3	6.41 (d, 9.6)
6	117.4	6.90 (d, 9.6)	117.4	6.89 (d, 9.6)	117.9	6.89 (d, 9.6)	117.4	6.90 (d, 9.6)
6a	138.5		139.3		139.3		138.1	
7	188.1		188.2		188.8		188.2	
7a	114.0		114.6		114.6		113.9	
8	158.1		158.5		158.5		158.1	
8-OH		12.27 (brs)		12.28 (brs)		12.20 (brs)		12.30 (brs)
9	138.4		138.5		138.5		138.0	
10	133.7	7.88 (d, 7.6)	133.7	7.88 (d, 7.6)	134.3	7.88 (d, 7.6)	133.7	7.87 (d, 7.6)
11	119.8	7.61 (d, 7.6)	119.8	7.61 (d, 7.6)	120.4	7.60 (d, 7.6)	119.1	7.60 (d, 7.6)
11a	130.5		130.6		131.1		130.4	
12	182.2		182.3		182.8		182.2	
12a	138.8		139.1		139.1		138.2	
12b	77.0		77.4		77.1		76.7	
12b-OH		4.67 (brs)		4.58 (brs)		4.55 (brs)		4.58 (brs)
13	26.5	1.47 (s)	25.8	1.45 (s)	27.1	1.46 (s)	25.8	1.44 (s)
1'	71.5	4.87 (dd, 1.4, 11.0)	71.2	4.95 (dd, 1.4, 11.3)	72.1	4.94 (dd, 2.1, 11.0)	70.5	4.86 (dd, 1.4, 11.3)
2'	38.9	1.39 (m) 2.55 (m)	36.7	1.40 (m) 2.43 (ddd, 2.1, 4.8, 13.1)	37.3	1.38 (m) 2.45 (m)	38.2	1.36 (m) 2.50 (m)
3'	71.7	3.90 (m)	77.1	3.80 (m)	69.9	3.81 (m)	70.8	3.79 (m)
3'-OH		4.28 (brs)						4.97 (brs)
4'	89.4	3.20 (m)	74.5	3.48 (t, 4.1)	75.1	3.47 (m)	88.4	3.04 (t, 8.4)
5'	74.7	3.56 (m)	74.6	3.56 (m)	75.2	3.55 (m)	73.9	3.54 (m)
6'	18.4	1.39 (d, 6.2)	17.5	1.38 (d, 6.8)	18.1	1.38 (d, 6.2)	18.5	1.34 (d, 6.0)
1''	95.2	5.37 (d, 3.4)	91.4	5.17 (d, 3.4)	92.0	5.17 (d, 2.8)	98.9	4.97 (brs)
2''	142.2	6.84 (dd, 3.4, 10.3)	71.0	4.33 (m)	71.8	4.32 (m)	23.8	1.67 (m) 2.09 (m)
3''	127.4	6.14 (d, 10.4)	40.0	2.62–2.66 (m), 2H	40.6	2.62–2.64 (m), 2H	24.6	1.96 (m) 2.08 (m)
4''	195.3		208.0		208.4		75.6	3.70 (brs)
5''	72.1	4.75 (q, 6.2)	77.8	4.71 (q, 6.8)	78.4	4.70 (q, 7.6)	67.3	4.22 (m)
6''	15.2	1.44 (d, 6.2)	16.9	1.39 (d, 6.2)	16.8	1.36 (d, 7.6)	16.5	1.26 (d, 6.8)
1'''	88.7	5.57 (d, 3.4)	92.8	5.40 (t, 6.2)	89.3	5.57 (d, 3.4)	95.8	5.25 (d, 3.6)
2'''	142.8	6.67 (dd, 3.4, 10.3)	28.3	2.16 (d, 3.4) 2.30 (m)	143.4	6.67 (dd, 3.4, 10.3)	142.8	6.87 (dd, 3.6, 10.1)
3'''	127.8	6.06 (d, 9.6)	33.4	2.38–2.40 (m), 2H	128.3	6.05 (d, 10.3)	127.5	6.04 (d, 10.1)
4'''	196.8		211.0		197.4		197.0	
5'''	70.7	4.72 (q, 6.8)	71.5	4.51 (q, 6.9)	71.3	4.74 (q, 7.6)	70.2	4.55 (q, 6.7)
6'''	15.1	1.43 (d, 6.8)	14.8	1.37 (d, 6.9)	15.7	1.42 (d, 6.9)	14.6	1.38 (d, 6.7)
1''''							92.8	5.40 (t, 6.3)
2''''							28.3	1.86 (m) 2.29 (m)
3''''							33.4	2.36–2.38 (m), 2H
4''''							210.9	
5''''							72.0	4.51 (q, 6.6)
6''''							14.8	1.39 (d, 6.6)

<sup>a</sup>Measured in CDCl<sub>3</sub>.

**Table 4** Biological activities of saprolmycin A (1)–E (5)

Test organisms	MIC ( $\mu\text{g ml}^{-1}$ )				
	1	2	3	4	5
<i>Saprolegnia parasitica</i>	0.0039	8	1	1	0.0078
<i>Staphylococcus aureus</i>	62.5	250	125	62.5	15.6
<i>Bacillus subtilis</i>	125	250	250	62.5	7.8
<i>Escherichia coli</i>	>1000	>1000	>1000	>1000	>1000
<i>Pseudomonas putida</i>	500	>1000	>1000	>1000	>1000
<i>Synechocystis</i> sp.	>100	>100	50	>100	>100
<i>Chlorella kessleri</i>	>100	>100	>100	>100	>100
<i>Saccharomyces cerevisiae</i>	>1000	>1000	>1000	>1000	>1000
<i>Phoma</i> sp.	>1000	>1000	>1000	>1000	>1000
<i>Daphnia pulex</i>	ND	ND	ND	ND	4.5 <sup>a</sup>

Abbreviation: ND, not determined.

<sup>a</sup>The value for *D. pulex* was determined as LD<sub>50</sub>.

in Figure 3 and the assignments of <sup>1</sup>H and <sup>13</sup>C NMR signals of **3** were demonstrated in Table 3.

**Saprolmycin B (2).** The ESI-MS spectrum of **2** showed a pseudo-molecular ion peak [M-H]<sup>-</sup> at *m/z* 707, the UV and IR data as shown in Table 1 closely resembled those of **5** and **6**.<sup>15</sup> The molecular formula of **2** was elucidated to be C<sub>37</sub>H<sub>40</sub>O<sub>14</sub> from its HR-FAB-MS data (found *m/z* 715.2603 [M+Li]<sup>+</sup>, calcd. *m/z* 715.2578 [M+Li]<sup>+</sup>), indicating that **2** was a dihydro derivative of **3**. Comparing the <sup>1</sup>H NMR spectra of **2** and **3**, we noted that the two olefinic proton signals of an aculose in **3** were not present in **2**; however, we did identify four aliphatic protons in **2**, namely H-2''' ( $\delta_{\text{H}}$  2.16 and  $\delta_{\text{H}}$  2.30) and H-3''' ( $\delta_{\text{H}}$  2.38–2.40, 2H), indicating the presence of a cinerulose. HMBC connections between the 2-oxygenated cinerulose and the aglycon pyran moieties were observed likewise in **3** indicated the formation of a dioxane ring. Therefore, we considered a cinerulose bonded to C-3 of the aglycon chromophore via a glycosidic bond and it was confirmed by the HMBC correlation (H-1''' ( $\delta_{\text{H}}$  5.40) to C-3 ( $\delta_{\text{C}}$  82.5)). The structure of **2** was determined as shown in Figure 3 and the total assignments of <sup>1</sup>H and <sup>13</sup>C NMR signals of **2** were demonstrated in Table 3.

### Biological activity

The five compounds discussed herein are new members of the angucycline antibiotic family, which comprises >100 microbial metabolites.<sup>18</sup> Angucycline compounds are reported to be bioactive substances and include anti-gram-positive bacterial, antitumor and enzyme inhibiting agents.<sup>18</sup> The biological activities of **1**–**5** were measured against *S. parasitica*, fungi, gram-positive and -negative bacteria, green and blue-green algae, and zooplankton. As demonstrated in Table 4, all five compounds showed weak antimicrobial activities against gram-positive bacteria (8–250  $\mu\text{g ml}^{-1}$ ) but were almost inactive against gram-negative bacteria and fungi (>1000 and 500  $\mu\text{g ml}^{-1}$ ). These activity profiles are similar to those of known angucycline antibiotics.<sup>19–21</sup> **2**, **3** and **4** inhibited the growth of *S. parasitica* at a concentration of 1–8  $\mu\text{g ml}^{-1}$ . Remarkably, **1** and **5**, which possess aculoses in their side chains, showed much higher activities against *S. parasitica* at much lower nanogram concentrations (3.9–7.8 ng ml<sup>-1</sup>). Their activities against green and blue-green algae and zooplankton were 10<sup>4</sup> to 10<sup>3</sup>-fold less than those against *S. parasitica*. Overall, these results clearly indicated that **1** and **5** exhibited high selective activities against *S. parasitica* and low

toxicities to environmental planktons, microalgae and zooplankton. Therefore, these compounds may be considered as potent and environmentally safe anti-saprolegniasis candidates for use in the aquaculture industry.

### ACKNOWLEDGEMENTS

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