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New Rugulosins, Anti-MRSA Antibiotics, Produced by *Penicillium radicum* FKI-3765-2

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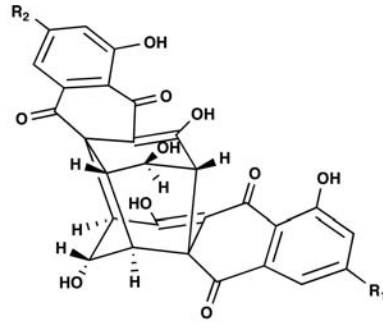
ABSTRACT



Rugulosin A (1): R_1 = -CH_3, R_2 = -CH_3
Rugulosin B (2): R_1 = -CH_3, R_2 = -CH_2OH
Rugulosin C (3): R_1 = -CH_2OH, R_2 = -CH_2OH

New rugulosins B (2) and C (3) were isolated together with known rugulosin (renamed rugulosin A in this paper, 1) from whole culture of *Penicillium radicum* FKI-3765-2, and their structures were elucidated by NMR spectroscopy. Rugulosins A and C were a homodimer of the same anthraquinone moieties, whereas rugulosin B was a heterodimer of analogous anthraquinone moieties. Rugulosins A to C showed antimicrobial activity against methicillin-resistant *Staphylococcus aureus*.

In a previous study, xanthoradones were discovered as potentiators of imipenem activity against methicillin-resistant *Staphylococcus aureus* (MRSA) from the whole culture of *Penicillium radicum* FKI-3765-2.[1] Further precise analysis of the metabolites led to the discovery of three structurally related compounds showing anti-MRSA activity. One was identified as rugulosin[2] (**1**, rugulosin A in this paper), whereas the others, named rugulosins B (**2**) and C (**3**), were new. Rugulosin A was originally isolated as a mycotoxin, whose structure was finally determined by Shibata and coworkers in the 1970s.[2]

Furthermore, rugulosin A was reported to exhibit antimicrobial activity[3] and anti-HIV activity.[4] Recently, total

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synthesis of rugulosin was also established.[5] In this study, the isolation, structure elucidation including absolute stereochemistry, and biological activity of **2** and **3** are described.

P. radicum FKI-3765-2 was fermented in rice medium at 27 °C for 15 days under static conditions.[1a] The whole culture (500 g) was treated with acetone, and the supernatant was concentrated under reduced pressure. The resulting aqueous layer was extracted three times with ethyl acetate. The organic layer was concentrated to give a dark brown oil (1.02 g). This oil was applied to an ODS column (100 g), and compounds **1** to **3** were eluted stepwise with 30%, 50%, 70%, and 100% CH_3CN containing 0.050% TFA (400 mL × 2 tubes each). The fraction (first tube of 70% CH_3CN) containing **1** was concentrated, and the resulting oil (95.2 mg) was purified with preparative HPLC under the following conditions: column, PEGASIL ODS (Senshu Sci. i.d. 20 × 250 mm); solvent, 70% CH_3CN containing 0.050% TFA; flow rate, 8.0 mL/min; detection, UV at 210 nm. The fraction eluted as a peak with a retention time of 17.3 min was collected and concentrated to give pure **1** as yellow crystals (41.4 mg). The 30% CH_3CN fractions containing **2** and **3** were concentrated to yield a red brown oil (101.9 mg). Compounds **2** and **3** were finally purified with preparative HPLC (column, PEGASIL ODS; solvent, 35% CH_3CN containing 0.050% TFA; flow rate, 8.0 mL/min; detection, UV at 210 nm). The respective fractions eluted as peaks at 36.9 and 15.9 min were collected and concentrated to give pure **2** (16.9 mg) and **3** (13.3 mg) as yellow crystals.

From various spectral data by FAB-MS (*m/z* 543 $(M + H)^+$), HRFAB-MS (molecular formula $C_{30}H_{22}O_{10}$), and NMR, the planar structure of **1** was confirmed to be a dimer of the emodin-type anthraquinone (6-methyl-1,3,8-trihydroxyanthraquinone, Figure 1), which was reported as a mycotoxin in 1925.[6] Two stereoisomers at C-3/C-3′ to the planar structure have been reported: rugulosin[2] isolated from various *Penicillium* and graciliformin (**4**)[7] isolated from lichens (Figure 1). The stereochemistry of **1** was deduced by comparing literature values in ^1H NMR spectra between rugulosin[2b] and **4**;[7] the chemical shifts at H-3/H-3′ of **1** were δ 4.38 with a coupling constant of $J_{2,3} = 6.0$ Hz, which were the same as those reported for rugulosin,[2b] while those of **4** were δ 4.90 with a coupling constant of $J_{2,3} = 0$ Hz.[7] Furthermore, cross peaks were observed between H-2/H-2′ (δ 2.77) and H-3/H-3′ (δ 4.38) and between H-3/H-3′ and H-4/H-4′ (δ 3.36), respectively, in ROESY experiments (Figure 2). These data indicated that **1** was identified with rugulosin (renamed rugulosin A herein).

Compound **3** had a molecular ion peak at *m/z* 575 $(M + H)^+$ in FAB-MS, and the molecular formula $C_{30}H_{22}O_{12}$ in HRFAB-MS [*m/z* 575.1192 $(M + H)^+$, Δ +0.2 mmu], requiring 20 degrees of unsaturation. IR absorption at 3421, 1712, and 1660 cm^{-1} indicated the presence of hydroxy and carbonyl groups. UV absorption at 247 nm indicated the presence of a quinone skeleton.[5b] These data showed that **3** has a skeleton similar to **1** and a bigger molecular formula by two oxygen atoms than **1**. The ^1H and ^{13}C NMR spectra of **3** revealed 9 proton and 15 carbon signals, which were classified into one sp^3 oxygenated methylene, two sp^3 methines, one sp^3 oxygenated methine, one sp^3 quaternary carbon, two sp^2 methines, four sp^2 quaternary carbons, two sp^2 oxygenated quaternary carbons, and two quinone carbonyl carbons by analysis of HSQC data. These data accounted for only half of the molecular formula, concluding that **3** should be the homodimer of a 15 carbon monomer. Comparison of NMR data between **1** and **3** indicated that the methyl protons (H_3-15, δ 2.41) in **1** are replaced with sp^3 oxygenated methylene protons (H_2-15, δ 4.60) in **3**. In fact, cross peaks were observed from H-6 (δ 7.57) to C-7

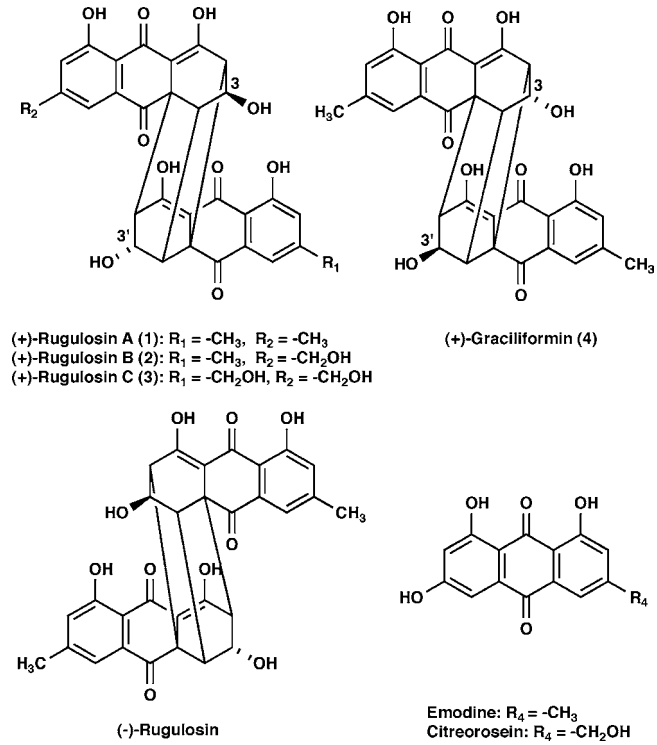

Figure 1. Structures of anthraquinone dimers and monomers.

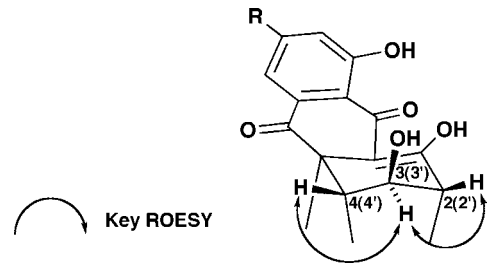

Figure 2. Key ROESY correlations of rugulosins.

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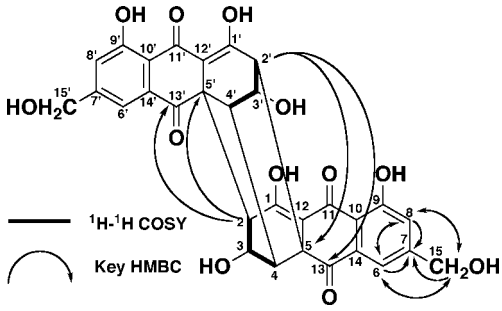

Figure 3. $^{1}H-^{1}H$ COSY and HMBC correlations of **3**.

(δ 152.2), C-8 (δ 120.7), and C-15 (δ 62.0); from H-8 (δ 7.27) to C-6 (δ 117.2), C-7, and C-15; and from H_2-15 (δ 4.60) to C-6, C-7, and C-8 in $^{13}C-^{1}H$ HMBC experiments (Figure 3). Additionally, the chemical shift of C-15 (δ 62.0) and the molecular formula showed the presence of a hydroxy group. Thus, the structure of the monomer was elucidated to be the citreorosein-type anthraquinone (6-hydroxymethyl-1,3,8-trihydroxyanthraquinones, Figure 1), which was also reported as mycotoxin in 1940.[8] Regarding the connection of the two monomers, the C-2-C-5′/C-2′-C-5 bonds between the monomers were defined by correlations from H-2/H-2′ (δ 2.76) to C-5′/C-5 (δ 55.5) and C-13′/C-13 (δ 194.0) in $^{13}C-^{1}H$ HMBC experiments, respectively. Furthermore, the third bond of C-4-C-4′ between the monomers should be formed because the two additional rings are required to satisfy the remaining 2 degrees of unsaturation. Taken together, the structure of **3** was elucidated as 15,15′-dihydroxy rugulosin A (designated rugulosin C, Figure 1).

All chemical shifts except for C-15 (C-15′) in **3** were comparable with chemical shifts reported for **1**.[2]

Compound **2** had physicochemical properties similar to those of **1** and **3**, indicating that they share a similar bisanthraquinone skeleton. HRFAB-MS [*m/z* 558.1240 (M + H)$^+$, Δ −0.9 mmu] of **2** indicated that the molecular formula $C_{30}H_{22}O_{11}$ is smaller by one oxygen atom than **3**, suggesting that **2** is a heterodimer consisting of two different anthraquinones. In fact, a slightly offset doubling of each signal was observed in the ^{1}H and ^{13}C NMR spectra. In addition, one methyl proton (H_3-15, δ 2.41) and one oxygenated methylene proton (H_2-15′, δ 4.60) were observed in the ^{1}H NMR spectrum of **2**, suggesting that **2** is composed of emodin-type[6] and citreorosein-type[8] anthraquinones. To confirm the structure, the $^{13}C-^{1}H$ HMBC experiment was carried out: (1) cross peaks were observed from H-6 (δ 7.44) to C-7 (δ 147.6), C-8 (δ 124.0), and C-15 (δ 21.5); from H-8 (δ 7.18) to C-6 (δ 120.5), C-7, and C-15; and from H_3-15 (δ 2.41) to C-6, C-7, and C-8 as expected in the emodin-type anthraquinone, and (2) cross peaks were observed from H-6′ (δ 7.58) to C-7′ (δ 152.2), C-8′ (δ 120.8), and C-15′ (δ 62.0); from H-8′ (δ 7.27) to C-6′ (δ 117.2), C-7′, and C-15′; and from H_2-15′ (δ 4.60) to C-6′, C-7′, and C-8′ as expected in the citreorosein-type anthraquinone. Moreover, the binding manner between the monomers was deduced to be similar to those of **1** and **3** from the correlation in the $^{13}C-^{1}H$ HMBC experiments (Figure 4). Taken together, the structure of **2** was elucidated as 15′-hydroxy rugulosin A (designated rugulosin B, Figure 1).

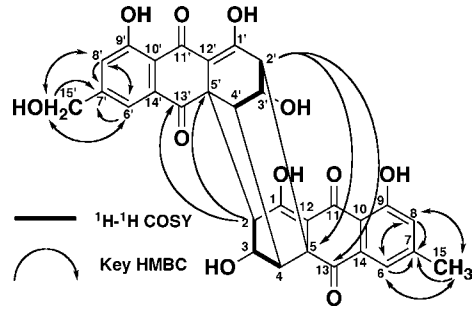

Figure 4. $^{1}H-^{1}H$ COSY and HMBC correlations of **2**.

The relative stereochemistry of **2** and **3** at C-3/C-3′ was deduced to be the same as that of **1** by similar ^{1}H NMR spectra and ROESY experiments (Figure 2). Furthermore, the absolute stereochemistry of **1** to **3** was concluded to be (+)-rugulosin A, (+)-rugulosin B, and (+)- rugulosin C by comparing the CD spectra of (+)-rugulosin and (−)-rugulosin;[2e] **1** to **3** showed negative cotton effects at 347−349 and 279−280 nm and positive cotton effects at 403−404 and 243 nm as well as (+)-rugulosin.[2e,9−11]

The anthraquinone monomers of **1** to **3**, emodine and citreorosein (Figure 1), were also isolated from the whole culture of *P. radicum* FKI-3765-2. Therefore, **1** to **3** might

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(11) Rugulosin A (**1**): yellow crystal; $[\alpha]^{22}_{D}$ +426.6° (*c* 0.1, dioxane); CD (dioxane) $\lambda_{ex\ termum}$ ($\Delta\varepsilon$) 243 (20.6), 280 (−30.6), 347 (−8.84), 403 (11.3); IR (KBr) υ_{max} 3434, 3411, 1689, 1616, 1569, 1481 cm^{-1}; UV (MeOH) λ_{max} 205 (ε 30075), 248 (ε 25569), 388 (ε 20575); FAB-MS *m/z* 543 (M + H)$^+$; HRFAB-MS *m/z* 543.1281 ((M + H)$^+$; calcd for $C_{30}H_{23}O_{10}$, 543.1291); ^{1}H NMR (DMSO-d_6) 14.7 (s, OH-1/OH-1′), 11.4 (s, OH-9/OH-9′), 7.44 (d, 1.2, H-6/H-6′), 7.18 (d, 1.2, H-8/H-8′), 4.38 (dd, 6.0, 2.3, H-3/H-3′), 3.36 (brs, H-4/H-4′), 2.77 (d, 6.0, H-2/H-2′), 2.41 (s, H-15/H-15′); ^{13}C NMR 194.6, 186.7, 181.7, 160.8, 148.3, 132.7, 124.7, 121.2, 114.8, 106.8, 69.2, 59.0, 56.3, 48.4, 22.2.

(12) Rugulosin B (**2**): yellow crystal; $[\alpha]^{22}_{D}$ +436.6° (*c* 0.1, dioxane); CD (dioxane) $\lambda_{ex\ termum}$ ($\Delta\varepsilon$) 243 (24.4), 280 (−34.1), 349 (−10.6), 404 (12.4); IR (KBr) υ_{max} 3423, 3266, 1727, 1720, 1652, 1454 cm^{-1}; UV (MeOH) λ_{max} 207 (ε 23289), 248 (ε 23453), 388 (ε 20879); ^{1}H and ^{13}C NMR data (Table 1); FAB-MS *m/z* 559 (M + H)$^+$; HRFAB-MS *m/z* 559.1240 ((M + H)$^+$; calcd for $C_{30}H_{23}O_{11}$, 559.1249).

(13) Rugulosin C (**3**): yellow crystal; $[\alpha]^{22}_{D}$ +289.9° (*c* 0.1, dioxane); CD (dioxane) $\lambda_{ex\ termum}$ ($\Delta\varepsilon$) 243 (25.1), 279 (−28.6), 347 (−7.73), 403 (14.6); IR (KBr) υ_{max} 3421, 2925, 1712, 1660, 1617, 1567 cm^{-1}; UV (MeOH) λ_{max} 207 (ε 28700), 247 (ε 31295), 390 (ε 27111); ^{1}H and ^{13}C NMR data (Table 1); FAB-MS *m/z* 575 (M + H)$^+$; HRFAB-MS *m/z* 575.1192 ((M + H)$^+$; calcd for $C_{30}H_{23}O_{12}$, 575.1190).

Table 1. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR Chemical Shifts of **2** and **3** (DMSO-d_6)

	rugulosin B (**2**)		rugulosin C (**3**)	
position	δ_c	δ_H	δ_c	δ_H
1	185.7		186.7	
1-OH		14.7 brs		14.7 brs
2	58.3 or 58.4[a]	2.77 m	58.5	2.76 d (J = 6.0 Hz)
3	68.5	4.38 m	68.5	4.38 dd (J = 6.0, 2.0 Hz)
3-OH		5.39 brs		
4	47.8	3.36 brs	47.8	3.37 brs
5	55.5 or 55.6[a]		55.5	
6	120.5	7.44 d (J = 1.2 Hz)	117.2	7.57 d (J = 2.0 Hz)
7	147.6		152.2	
8	124.0	7.18 d (J = 1.2 Hz)	120.7	7.27 d (J = 2.0 Hz)
9	160.1		160.1	
9-OH		11.4 s		11.4 s
10	114.1		114.9	
11	180.0		181.3	
12	106.1 or 106.2[a]		106.8	
13	193.9		194.0	
14	132.0		132.1	
15	21.5	2.41 s	62.0	4.60 s
1′	186.7			
1′-OH		14.7 brs		
2′	58.3 or 58.4[a]	2.77 m		
3′	68.5	4.38 m		
3′-OH		5.39 brs		
4′	47.8	3.36 m		
5′	55.5 or 55.6[a]			
6′	117.2	7.58 d (J = 1.2 Hz)		
7′	152.2			
8′	120.8	7.27 d (J = 1.2 Hz)		
9′	160.1			
9′-OH		11.4 s		
10′	114.9			
11′	180.7			
12′	106.1 or 106.2[a]			
13′	194.0			
14′	132.1			
15′	62.0	4.60 s		

[a] Indicates that assignment between monomers is interchangeable.

be artifacts in the process of the purification procedures. However, no products were produced by the routine treatment of emodine and citreorosein, and the contents of these monomers and dimers did not drastically change in the purification process from the culture extracts of *P. radicum* FKI-3765-2. Therefore, we concluded that compounds **1** to **3** were secondary metabolites of the fungus.

Although **1** was reported to have antimicrobial activity,[3] its anti-MRSA activity has not been reported. In this study, **1** to **3** were found to have anti-MRSA activity by the paper disk method, but no cytotoxic effects on Jurkat cells (a human T-cell leukemia-derived cell line) were observed even at 60−100 μg/mL. Therefore, anti-MRSA activity of **1** to **3** was confirmed by the liquid microdilution method.[9] Compound **1** showed potent antimicrobial activity against MRSA K-24[10] with an MIC value of 0.125 μg/mL, while **2** and **3** showed weak anti-MRSA activity with MIC values of 32 and 64 μg/mL, respectively. Taking the structural differences into consideration (Figure 1), the presence of a hydroxy group at C-15 (or/and C-15′) in **2** and **3** might be unfavorable for anti-MRSA activity.

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Supporting Information Available: NMR data of rugulosins B (**2**) and C (**3**). This material is available free of charge via the Internet at http://pubs.acs.org.

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