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FUROSONIN, A NOVEL HYDROLYZABLE TANNIN FROM *GERANIUM THUNBERGII* †

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Abstract – Furosonin (**2**), a novel hydrolyzable tannin, was isolated from *Geranium thunbergii* (Geraniaceae) leaves, and the structure was determined based on spectroscopic data. The effects of geraniin (**1**), furosonin (**2**), and related hydrolyzable tannins on antibiotic resistance were examined, and repandusinic acid A (**4**) was found to suppress oxacillin resistance of methicillin-resistant *Staphylococcus aureus*.

Aboveground parts of *Geranium thunbergii* (Geraniaceae, “gen-no-shouko” or “fuu-ro-sou” in Japanese) have been used to treat diarrhea and constipation as a Pharmacopoeia medicine in Japan. This plant is known to be rich in tannin. The major constituent, geraniin (**1**),[1] and structurally related hydrolyzable tannins, such as didehydrogerniin, furosinin, furosin,[2] geraniinic acids B and C,[3] and elaeocarpusin (ascorgeraniin),[4,5] were isolated from the plant. We have isolated furosonin (**2**), a tannin from *Geranium thunbergii* with a novel acyl group. In this study, we discuss the isolation and structure of **2**. Among the tannins structurally related to **1** and **2**, corilagin (**3**)[6] and repandusinic acid A (**4**)[7] suppressed oxacillin resistance of methicillin-resistant *Staphylococcus aureus* (MRSA).

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† Dedicated to Professor Ei-ichi Negishi, Purdue University, on the occasion of his 77th birthday.
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Dried leaves from *G. thunbergii*, cultivated in Okayama University Medicinal Botanical Garden, were homogenized in 70% acetone, and the concentrated filtrate from the homogenate was extracted with Et_2O, EtOAc, and *n*-BuOH, successively. Although analysis of the EtOAc extract identified **1** as the major constituent, the *n*-BuOH extract contained unidentified compounds. The *n*-BuOH extract was thus chromatographed on Toyopearl HW-40C and MCI-gel CHP-20P. Further purification using high-performance liquid chromatography (HPLC) yielded **2**, together with acalyphidin M_1 (**5**).[8]

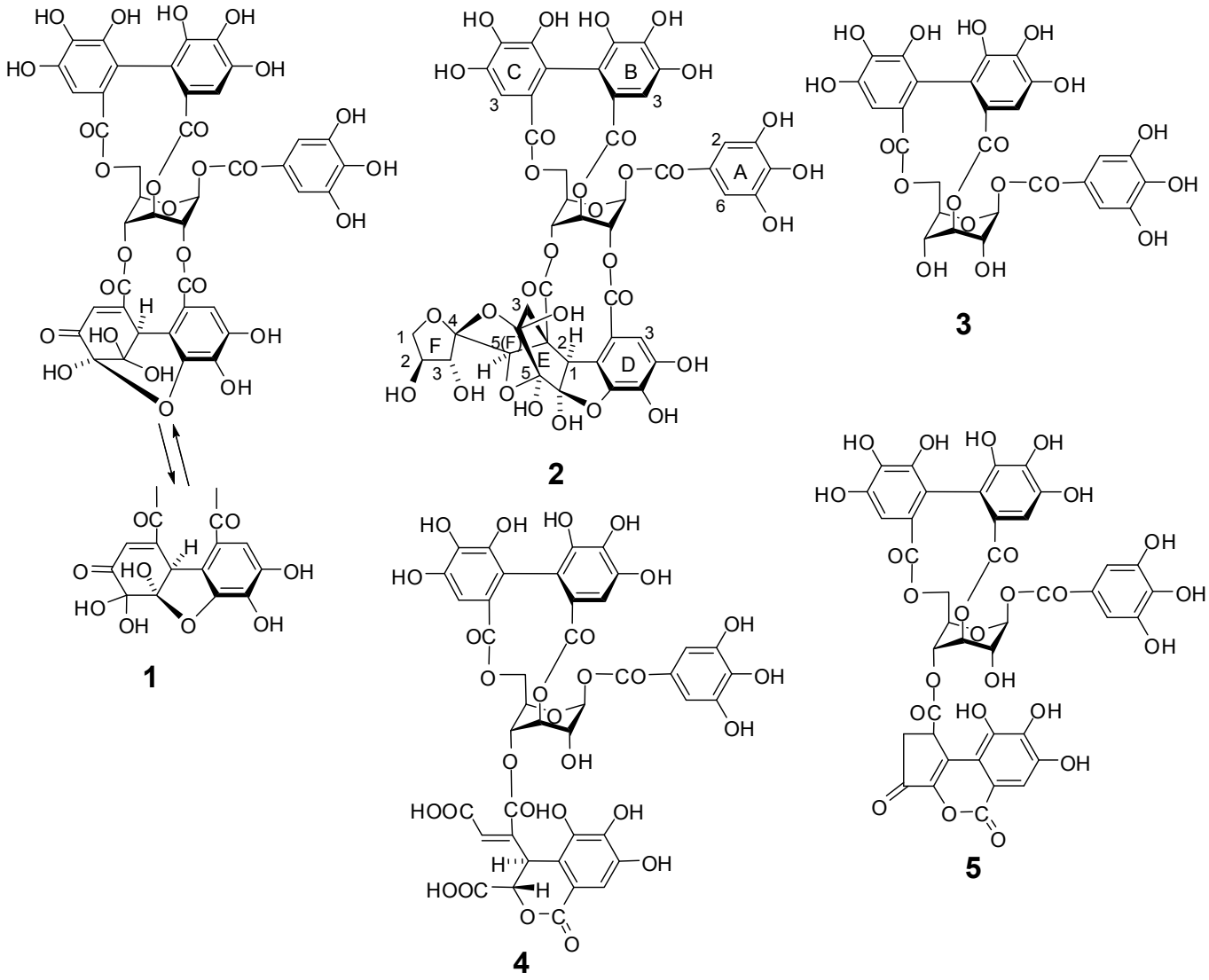

Figure 1. Structures of hydrolyzable tannins; geraniin (**1**), furosonin (**2**), corilagin (**3**), repandusinic acid A (**4**), and acalyphidin M_1 (**5**)

Furosonin (**2**) was obtained as a pale-yellow amorphous powder. High-resolution electrospray ionization mass spectrometry (HR-ESIMS) in negative-ion mode showed the $[M-H]^-$ ion at *m/z* 1083.1163, with the

molecular formula $C_{46}H_{36}O_{31}$ (calculated for $C_{46}H_{36}O_{31}$-H, 1083.1168). The ^{1}H NMR spectrum showed signals of a 2H singlet assignable to a galloyl group (δ 7.14), two 1H singlets from a hexahydroxydiphenoyl (HHDP) group (δ 6.60 and 7.02), and an additional aromatic singlet which was attributed to a penta-substituted benzene ring (δ 7.27) (unit D in formula **2**) in the aromatic proton signal region. On the other hand, the spectrum showed signals ascribed to $^1C_4$ glucopyranose core protons [δ 6.47 (br s, H-1), 5.54 (br s, H-2), 5.61 (br m, H-3), 5.28 (br m, H-4), 4.84 (t-like, *J*=10 Hz, H-5), 4.70 (dd, *J*=10, 11 Hz, H-6), and 4.38 (dd, *J*=8, 11 Hz, H-6)]. In the upper field region of the spectrum, methylene [δ 2.68 and δ 1.58 (each d, *J*=14 Hz, H-3 of unit E)] and methine [δ 4.68 (s), H-1 of unit E] protons with a long-range coupling were observed by ^{1}H-^{1}H correlation spectroscopy (COSY). In addition, the spectrum also showed signals of a methylene [δ 3.86 (dd, *J*=2.5, 9.5 Hz) and 4.12 (dd, *J*=5.5, 9.5 Hz) (H-1 of unit F)] – methine [δ 4.07 (ddd, J=1.5, 2.5, 5.5 Hz, H-2)] – methine [δ 4.15 (d, *J*=1.5 Hz, H-3)] system, as well as an isolated methine proton at δ 4.98 (s, H-5). These five protons, forming a pattern similar to that of corresponding protons in putranjivain A (**6**),[9] were suggestive of a unit F structure, containing a five-membered ring derived from ascorbic acid. These D, E, and F units were assigned as a novel acyl group.

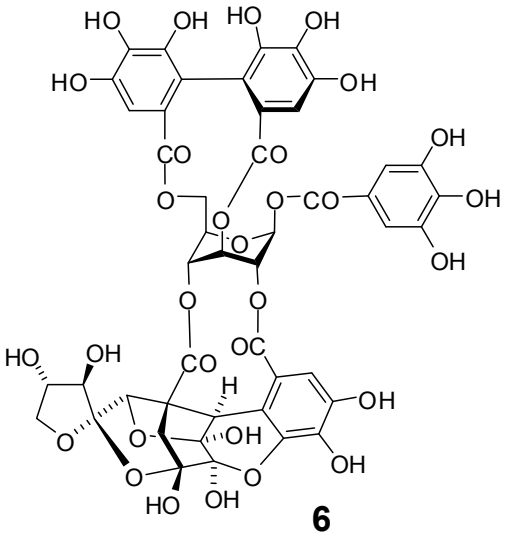

Figure 2. Structure of putranjivain A (**6**)

The presence of these constituent units was corroborated by the ^{13}C NMR spectrum. Galloyl group signals were observed at δ 119.9 (C-1), 110.5 (C-2, C-6), 145.9 (C-3, C-5), 139.9 (C-4), and 165.2 (C-7). The presence of an HHDP group was shown by the seven pairs of signals at δ 115.3, 117.0 (C-1), 124.4, 125.4 (C-2), 107.6, 110.2 (C-3), 144.6, 145.1 (2 × C), 145.3 (C-4, C-6), 136.3, 137.6 (C-5), 166.3, and 168.6 (C-7). Glucose carbon signals were observed at δ 91.7 (C-1), 70.4 (C-2), 62.9 (C-3), 66.1 (C-4), 73.4 (C-5), and 64.0 (C-6), corresponding to its $^1C_4$ conformation.[10]

Novel acyl group signals appeared as follows. Unit D signals were observed at δ 111.5 (C-1), 119.3 (C-2) (quaternary carbons), 114.5 (hydrogen-bearing C-3), 145.9 (C-4), 138.6 (C-5), 144.9 (C-6) (oxygen-bearing quaternary carbons), and 165.8 (ester carbonyl C-7), corresponding to the C-substituted galloyl structure. The downfield shift of the C-5 signal, relative to the corresponding carbon signals of HHDP (δ 136.3 and 137.6), was ascribed to the formation of the ether linkage at C-6. Unit F signals containing the furanose-like five-membered ring were observed at δ 75.3 (ether oxygen-bearing methylene C-1), 77.3 (C-2), 81.4 (C-3), 109.7 (hemi-ketal C-4), and 77.1 (C-5). Among them, C-2, C-3, and C-5 were oxygen-bearing methine carbons. The remaining signals attributed to unit E carbons were

observed at δ 52.0 (C-1 binding to the phenyl of unit D), 53.2 (C-2 binding to CO), 32.4 (methylene C-3), 98.4 (C-4), 98.9 (C-5), 98.8 (C-6) (hemi-ketal or *gem*-diol carbons), and 170.6 (C-7) (ester carbonyl). Assignments of the ester carbonyl groups of the acyl groups and their locations on the glucose core were attained based on the ^{1}H-^{13}C heteronuclear multiple-bond correlation spectroscopy (HMBC), as follows (Figure 3). The galloyl 2H singlet of H-2 and H-6 protons at δ_H 7.10 showed connectivity with glucose H-1 via the ester carbonyl signal at δ_C 165.2. The HHDP 1H singlets of H-3 protons at δ_H 7.00 and δ_H 6.60 were respectively connected with glucose H-3 and H-6 via ester carbonyl carbons at δ_C 166.3 and δ_C 168.6. The remaining glucose H-2 and H-4 were connected respectively with H-3 of unit D via an ester carbonyl carbon at δ_C 165.8 and with H-1 of unit E via an ester carbonyl carbon at δ_C 170.6.

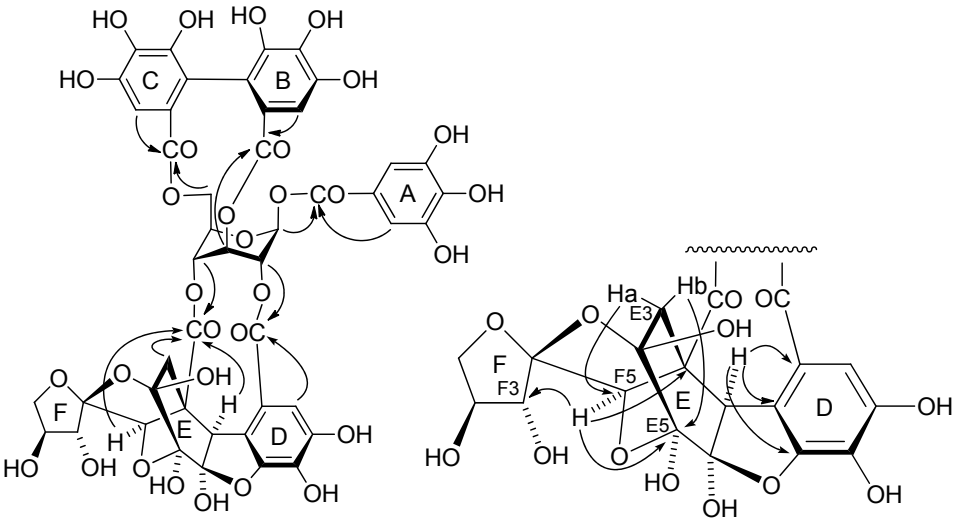

Figure 3. Key HMBC correlations observed for **2**

Linkages between units D and E were also corroborated by the HMBC correlations. H-1 of unit E showed correlations with unit E (C-2, C-3, and C-6) and unit D carbons (C-1, C-2, and C-6), in addition to C-7 of unit E. Furthermore, H-3a of unit E correlated with C-5 of unit F, along with unit E carbons (C-1, C-2, and C-4). On the other hand, H-3b of unit E correlated with C-1, C-2, and C-5 of unit E, which supported assignments of the unit E structure. H-5 of unit F correlated with C-2, C-5, and C-7 of unit E, in addition to C-3 of unit F, which also satisfied the linkages between units E and F.

The ROESY spectrum showed a correlation between H-1 in unit E with glucose H-1, reflecting the spatial proximity between these two protons, which satisfied the orientation of the acyl group on O-2 – O-4 of the glucose core (Figure 4). The ROESY spectrum also showed a correlation in unit

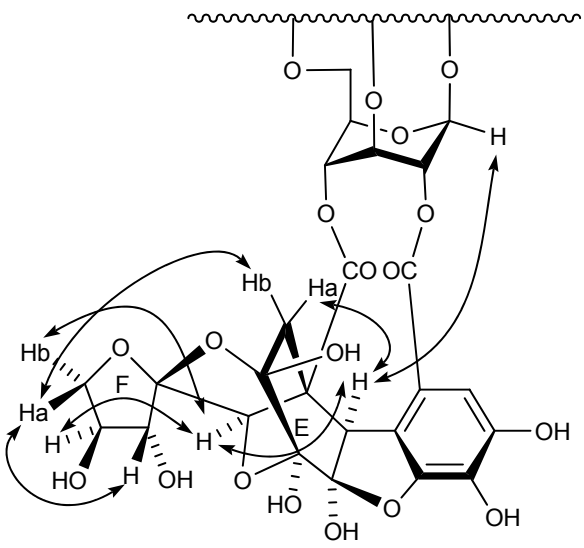

Figure 4. Key ROESY correlations observed for **2**

E H-1 and H-3a, which allowed us to discriminate between H-3a and H-3b of unit E. This correlation suggested that H-3a and H-3b were oriented towards units D and F, respectively. H-3b of unit E also showed an ROE correlation with H-1a of unit F, indicating that this H-1a proton was at the front side of the five-membered ring of unit F. In turn, H-1a of unit F showed an ROE correlation with H-3 (unit F).

H-1b – H-5 and H-2 – H-5 correlations were also observed among the unit F protons. Thus, the configurations on the carbons C-2 – C-5 of unit F were assigned as shown in the structural formulae.

A negative Cotton effect at 246 nm ([θ] -4.1 × 10^4) in the circular dichroism spectrum was indicative of an *R*-configuration of the biphenyl moiety of the B – C HHDP units.[11] Structure **2** was thus assigned to furosonin.

MRSA, which often acquires multi-drug resistance, causes serious clinical problems in hospitals, and identifying compounds that suppress drug resistance is an important strategy for treatment of infectious diseases. Since various tannins and related polyphenols,[12-15] including **3**,[7] are known to suppress β-lactam resistance, we examined the effects of **1**, **2** and structurally related tannins on MRSA antibiotic resistance. Since **3** and **4** were easily obtained from **1**, we also examined these compounds. Efflux pumps play an important role in multi-drug resistance, and therefore we examined the effects of tannins on norfloxacin.[16,17]

Table 1. Effects of tannins structurally related to geraniin (**1**) and furosonin (**2**) on minimum inhibitory concentrations (MIC) of oxacillin and norfloxacin against methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates.

	MIC (μg/mL)			
	Oxacillin OM623 [a)]	Norfloxacin OM623 [a)]	Norfloxacin OM584 [a)]	Norfloxacin OM481 [a)]
Oxacillin or Norfloxacin alone	256	32	64	128
Tannins added [b)]				
plus geraniin (**1**) (64μg/mL)	- [c)]	16	32	128
plus geraniin (**1**) (32μg/mL)	128			
plus furosonin (**2**) (64μg/mL)	- [c)]	32	32	128
plus furosonin (**2**) (32μg/mL)	256			
plus corilagin (**3**) (32μg/mL)	- [c)]	32	32	128
plus corilagin (**3**) (16μg/mL)	<1			
plus repandusinic acid A (**4**) (256μg/mL)	- [c)]	32	32	128
plus repandusinic acid A (**4**) (128μg/mL)	8			
plus acalyphidin M_1 (**5**) (128μg/mL)	- [c)]	32	32	64
plus acalyphidin M_1 (**5**) (64μg/mL)	64			

a) Clinical isolates of MRSA from Okayama University Hospital.
b) MIC of each of the tannins: **1**, 128 μg/mL; **2**, 128μg/mL; **3**, 64μg/mL; **4**, 512μg/mL; **5**, 256μg/mL.
c) Not tested.

The results were summarized in Table 1. All tannins except for **2** decreased the MIC of oxacillin against the MRSA OM623 strain at 1/4 MIC concentrations. Compounds **3** and **4** decreased the MIC of oxacillin from 256 to 8 μg/mL (for **4**) or <1 μg/mL (for **3**). On the other hand, the tannins did not decrease the MIC of norfloxacin noticeably. Further studies are required to identify additional compounds that suppress MRSA drug resistance.

EXPERIMENTAL

Specific rotations were recorded on a JASCO DIP-1000 digital polarimeter. CD spectra were measured on a JASCO J-720W spectrophotometer. ESI-MS was recorded on a Bruker Daltonics MicrOTOF II instrument in negative ion mode. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA AS600 spectrophotometer (600 MHz for ^{1}H, and 150.8 MHz for ^{13}C) at 300 K. Chemical shifts are given in δ (ppm) values relative to that of the solvent signal [acetone-d_6 (δ_H 2.04; δ_C 29.8)] on the tetramethylsilane scale. MRSA strains used in this study were clinical isolates from Okayama University Hospital.

Isolation of 2: Dried *Geranium thunbergii* leaves (200 g), cultivated in Okayama University Medicinal Botanical Garden, were homogenized with 70% aq. acetone (700 mL × 3). The homogenate was filtered and the filtrate was concentrated to 600 mL and extracted with Et_2O (600 mL × 3), EtOAc (600 mL × 3) and *n*-BuOH (600 mL × 3), successively. A part (3.6 g) of *n*-BuOH extract (13 g) was subjected to column chromatography over Toyopearl HW-40C with 70% aq. EtOH and the eluate was monitored by HPLC. Combined fractions 46-53 (134.4 mg), which showed HPLC peaks representing unidentified compounds, were purified by column chromatography on MCI-gel CHP-20P with aqueous MeOH. Combined fractions 46-61 (11.2 mg, eluted with 20% aq. MeOH) from the MCI-gel column were purified by preparative HPLC to yield **2** (3.6 mg) under the following conditions: Column, YMC-Pack ODS-A A-324 (YMC) column (10 i.d. × 300 mm); solvent, 0.01 M H_3PO_4, 0.01 M KH_2PO_4, and MeOH (11:11:3; flow rate, 2 mL/min; 280 nm UV detection); column temperature, 40 °C. Fractions 10-20 (15.2 mg, eluted with 40% MeOH) from the MCI-gel chromatography were separated on a Sep-Pak (Plus) C18 cartridge to yield **5** (5.6 mg, eluted with 30% aq. MeOH). Similarly, the residual (9.4 g) *n*-BuOH extract was purified by column chromatography on Toyopearl HW-40, MCI-gel CHP-20P, and Sephadex LH-20, and the fraction containing **2** was purified by preparative HPLC to yield **2** (12.5 mg).

Compound 2: Pale-yellow amorphous powder, $[\alpha]_D$ - 33.3 (*c* 1.0, MeOH). HR-ESI MS *m/z*: 1083.1163 $[M - H]^-$ (calculated for $C_{46}H_{35}O_{31}$, 1083.1168). UV λ_{max} (MeOH) nm (log ε): 220 (4.97), 279 (4.60). CD (MeOH) [θ] (nm): $+3.5 \times 10^4$ (200, shortest wavelength measured), -2.8×10^4 (222), $+1.4 \times 10^4$ (236), -4.1×10^4 (246), $+1.0 \times 10^4$ (263), -4.1×10^4 (289).

Preparation of 3 and 4 from 1: Compound **1** (100 mg) was dissolved in phosphate buffer (pH 7.4, 50 mL), and the solution was maintained at 40 °C for 16 h. After acidifying the solution to end the reaction, the solution was extracted with ethyl acetate. The EtOAc extract was applied to column chromatography on Toyopearl HW-40C to yield **3** (9.3 mg), and the aqueous layer was subjected to an MCI-gel column chromatography to produce **3** (14.9 mg) and **4** (6.3 mg), which were identified based on their ^{1}H NMR spectra.

Effects of tannins on MIC of oxacillin and norfloxacin: The MIC of antimicrobial agents were determined using the broth dilution method.[15] Briefly, an inoculum of about 10^5 CFU in 100 μL of Mueller–Hinton broth (Difco) supplemented with 0.85% NaCl were incubated in 96-well microtiter plates at 35 °C for 24 h. The lowest concentration of each of the antibiotics or tannins where the visual turbidity was low after incubation was considered to be the MIC.

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