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NOTE

Aquastatin C, a new glycoaromatic derivative from *Sporothrix* sp. FN611

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Bacterial fatty acid synthesis (FAS) is an attractive antibacterial target, as FAS is organized differently in bacteria and mammals.[1,2] Fatty acid biosynthesis in bacteria is crucial for the production of a number of lipid-containing components, including the cell membrane. The bacterial fatty acid system (FAS II) uses discrete monofunctional enzymes, which operate in conjunction with acyl carrier protein (ACP)-associated substrates, whereas mammalian fatty acid synthase (FAS I) is mediated by a single multifunctional enzyme–ACP complex. The differences in prokaryote and eukaryote fatty acid biosynthesis provide an attractive opportunity for selective FAS II inhibition, which is a potential strategy for the development of antibacterial agents. Bacterial enoyl-ACP reductase, which catalyzes the final and rate-limiting step in type II FAS, has been validated as a novel target for the development of antibacterial drugs.[3,4] Three isoforms, FabI, FabK and FabL, have been detected in enoyl-ACP reductase. Most of bacteria including *Staphylococcus aureus* contain FabI, but *Streptococcus pneumonia* has FabK. *Enterococcus faecalis* and *Pseudomonas aeruginosa* were known to contain both FabI and FabK, whereas *Bacillus subtilis* contains both FabI and FabL. InhA is the FabI homolog from *Mycobacterium tuberculosis*. Indeed, the antibacterial target of triclosan[5] and isoniazid,[6] which are currently used antibacterial agents, have been determined to be the FabI and InhA, respectively. Therefore, the enoyl-ACP reductase inhibitors may prove to be interesting lead compounds for the development of effective antibacterial drugs.

In the course of our screening for inhibitors of bacterial enoyl-ACP reductase from microbial metabolites, we previously isolated aquastatin A (**2**) from a fungal strain *Sporothrix* sp. AT 28.[7] Aquastatin B (**3**) was obtained by acid hydrolysis from aquastatin A. Further HPLC studies on minor and less polar metabolites with a similar UV spectra have resulted in isolation of a new derivative of aquastatin A, aquastatin C (**1**; Figure 1). In this paper, we report the isolation and structure determination of **1**, and the structure–activity relationship.

ISOLATION

The ethylacetate extracts from the culture broth (6 l) of *Sporothrix* sp. AT 28 was subjected to silica gel (56×200 mm, Merck art no. 7734.9025, Merck, Darmstadt, Germany) column chromatography followed by stepwise elution with $CHCl_3$–MeOH (10:1, 5:1, 2:1, 1:1). The active fractions eluted with $CHCl_3$–MeOH (2:1) were pooled and concentrated *in vacuo*. The HPLC analysis of the residue dissolved in MeOH showed the presence of another peak with a similar UV spectra with that of **2**. The peak was purified by HPLC equipped with Waters 510 HPLC pump and Waters 996 Photodiode Array Detector (Waters, Milford, MA, USA). The reverse phase HPLC column (20×250 mm, YMC C_{18}) was eluted with CH_3CN–H_2O (80:20) at a flow rate of 8 ml min^{-1} to afford 4.2 mg of **1** with a retention time of 49.2 min as a white powder. Compound **2** was detected at a retention time of 7.5 min by HPLC in the same condition.

STRUCTURE ELUCIDATION

The molecular formula of **1** was determined to be $C_{29}H_{48}O_9$ on the basis of high-resolution ESI-MS ($(M+Na)^+$ at *m/z* 563.3192 (+0.1 mmu error), $(M-H)^-$ at 539 *m/z*) in combination with ^{1}H- and ^{13}C-NMR data. The IR data suggested the presence of a carbonyl (1646 cm^{-1}) and a hydroxyl (3428 cm^{-1}) moiety (Table 1).

The ^{1}H-NMR data (Table 2) with the COSY spectrum suggested the presence of two aromatic protons attributable to an 1, 2, 4, 6-tetrasubstituted benzene ring at δ 6.48 (d, J=2.4) and δ 6.45 (d, J=2.4), protons attributable to a hexopyranoside moiety from δ 4.89 (1′-H) to δ 3.76 and δ 3.77, a methoxy proton at δ 3.91 (s) and protons attributable to a typical aliphatic chain from δ 2.80 to δ 0.86. In the ^{13}C-NMR spectrum, together with the HMQC spectrum, two olefinic carbons, 4 sp^2 quaternary carbons, a carbonyl carbon, a methoxy carbon, an anomeric carbon, 4 hydroxylated methine carbons, a hydroxylated methylene carbon, 14 aliphatic methylene carbons and an aliphatic methyl carbon were observed. The vicinal coupling constants together with the chemical shifts of ^{13}C-NMR in

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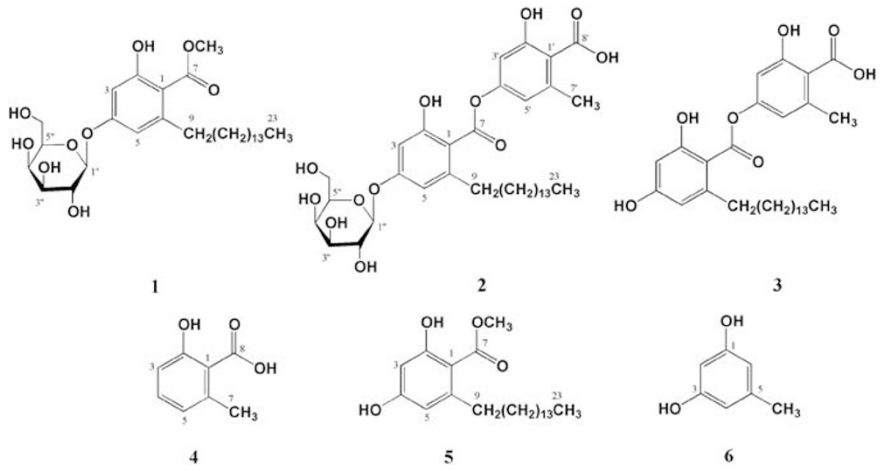

Figure 1 Chemical structures of aquastatins C (**1**), A (**2**), B (**3**) and related compounds.

Table 1 Physicochemical properties of 1

Appearance	*White powder*
$(\alpha)_D$	−1.3 (*c* 0.35, MeOH)
ESI-MS (*m/z*)	539 $(M-H)^-$, 563 $(M+Na)^+$
HRESI-MS (m/z)	
Found.	563.3192
Calcd.	563.3191 $(M+Na)^+$
Molecular formula	$C_{29}H_{48}O_9$
UV λ_{max}nm(log ε) (MeOH)	214 (4.54), 257 (4.06), 302 (3.58)
IR (KBr)νcm^{-1}	3428, 2921, 1646, 1621, 1263, 1086

Abbreviation: HRESI-MS, high-resolution electron spray ionization-MS.

the sugar signals indicated the presence of the β-galactopyranoside. The methoxyl protons at δ 3.91 (H-8) were long-range coupled with the carbonyl carbon at δ 172.3 (C-7) in the HMBC spectrum, indicating the carboxylic acid methyl ester group. In the ^{1}H–^{1}H COSY spectrum, a triplet methyl signal at δ 0.86 (H-23) and two multiplet methylene signals at δ 2.80 (H-9) and δ 1.50 (H-10) were correlated with a group of methylene signals at δ 1.24 (H-11 to H-22). In the ^{13}C-NMR data, a group of methylene carbons at δ 30.0–δ 37.1 together with the molecular formula indicated that the aliphatic chain is the pentadecyl group. These spectral data with the molecular formula indicated that four substituents in the 1, 2, 4, 6-tetrasubstituted benzene ring are a β-galactopyranoside, a pentadecyl, a carboxylic acid methyl ester and a hydroxyl. The position of the four substituents was determined by the HMBC and NOESY spectrum (Figure 2). The anomeric proton of the β-galactopyranoside at δ 4.89 (H-1″) showed a long-range correlation with the sp^2 quaternary carbon at δ 162.1 (C-4), which was in turn long-range correlated with the two meta-coupled aromatic protons at δ 6.48 (H-3) and δ 6.45 (H-5). In addition, H-1′ showed NOEs with both H-3 and H-5. This data clearly indicated that the sugar was attached with C-4. The methylene protons at δ 2.80 (H-9) of the pentadecyl group showed HMBC correlations with C-5 and two sp^2 quaternary carbons at δ 108.1 (C-1) and δ 148.2 (C-6). In addition, the long-range correlations from H-5 to C-1, C-3, C-4 and C-9 and from H-3 to C-1, C-2, C-4 and C-5 were observed. These spectral data indicated that the carboxylic acid methyl ester, the hydroxyl and the pentadecyl group were attached with C-1, C-2 and C-6, respectively. Thus, the structure of **1** was determined to be a new glycoside of 2-hydroxy-6-pentadecyl benzoic acid methyl ester as shown in Figure 1.

Acid hydrolysis of compound 2

Compound **2** (47 mg) dissolved in 0.4 ml MeOH was treated with HCl (2.4 ml) and then stirred overnight at room temperature. The resulting hydrolysate was then extracted twice with ethyl acetate (20 ml) and the extract was concentrated *in vacuo*. The residue dissolved in MeOH was applied to SiO_2 TLC developed with $CHCl_3$–MeOH (7:1) to yield **5** (2.8 mg) and **6** (2.9 mg) at R_f of 0.80 and 0.50, respectively.

Compound **5**: $C_{23}H_{38}O_4$: a white powder. ^{1}H-NMR (500 MHz, CD_3OD) δ: 6.18 (1H, d, 2.5), 6.14 (1H, d, 2.5), 3.88 (3H, s), 2.79 (2H, t, 8.0), 1.52 (2H, m), 1.29 (24H, m), 0.89 (3H, t, 7.2). ESI-MS: 377.8 $(M-H)^-$.

Compound **6**: $C_7H_8O_2$: a white powder. ^{1}H-NMR (500 MHz, CD_3OD) δ: 6.11 (2H, d, 2.0), 6.05 (1H, d, 2.0), 2.17 (3H, s); ^{13}C-NMR (125 MHz, CD_3OD) δ: 159.4 (C-1 and C-3), 141.2 (C-5), 108.7 (C-4 and C-6), 100.8 (C-2), 21.7 (C-7). ESI-MS: 123.4 $(M-H)^-$.

Aquastatin C is a new derivative of aquastatin A, in which the 2-hydroxy-6-methyl benzoic acid ring of aquastatin A was deleted. Aquastatin C could be a direct fermentation product, not artifact that was hydrolyzed by some kind of esterase from the producing strain, because derivatives of aquastatin C with an unsaturated acyl group instead of the pentadecyl were also detected even though the exact position of the double bond was not determined. Aquastatin A and its related compounds KS-501 and KS-502 have a common structure, in which two aromatic rings are linked by an ester bond, and a sugar and a pentadecyl group are attached with the ring.[8–10] Aquastatin C is a rare metabolite incorporating one aromatic ring in its structure. Aquastatin B is a deglycosylated product derived from acid hydrolysis of aquastatin A.[8] Previously aquastatins A and B were reported to strongly inhibited bacterial enoyl-ACP reductases including *S. aureus* FabI and *S. pneumoniae* FabK in a dose-dependent manner.[7] Also aquastatins A and B were reported to exert antibacterial activity against the Gram-positive pathogens, including *S. aureus*, methicillin-resistant *S. aureus* and *S. pneumonia*. However, an active moiety for anti-enoyl-ACP reductases activity of aquastatins A and B has not been determined yet.

Effect of aquastatin C against enoyl-ACP reductases and bacterial growth was investigated according to the previously reported method.[11] Interestingly, aquastatin C did not inhibit *S. aureus* FabI and *S. pneumoniae* FabK even at 100 μM (Table 3). Consistent with no

Table 2 ^{1}H and ^{13}C NMR spectral data for 1 and 2

	1		*2*	
Position	δ_H *(multiplicity,* J_{HH}*)*	δ_c	δ_H *(multiplicity,* J_{HH}*)*	δ_c
1		108.1 C		112.2 C
2		163.6 C		162.9 C
3	6.48 (1H, d, 2.4)	102.5 CH	6.47 (1H, d, 2.1)	102.9 CH
4		162.1 C		164.0 C
5	6.45 (1H, d, 2.4)	112.0 CH	6.43 (1H, d, 2.1)	109.5 CH
6		148.2 C		148.3 C
7		172.3 C		170.2 C
8	3.91 (3H, s)	52.3 CH_3		
9	2.80 (2H, m)	37.1 CH_2	2.88 (2H, m)	37.2 CH_2
10	1.50 (2H, m)	32.6 CH_2	1.56 (2H, m)	33.3 CH_2
11–22	1.24 (24H, m)	32.6–30.0 CH_2	1.19 (24H, m)	33.0–30.5 CH_2
23	0.86 (3H, t, 6.6)	14.2 CH_3	0.81 (3H, t, 5.0)	14.4 CH_3
1′				118.1 C
2′				164.7 C
3′			6.51 (1H, d, 2.4)	108.2 CH
4′				153.0 C
5′			6.39 (1H, d, 2.4)	115.5 CH
6′				144.6 C
7′			2.59 (3H, s)	23.7 CH_3
8′				175.7 C
1″	4.89 (1H, d, 7.5)	101.4 CH	4.92 (1H, d, 7.2)	102.0 CH
2″	3.78 (1H, m)	71.6 CH	3.82 (1H, m)	72.0 CH
3″	3.57 (1H, dd, 9.6, 3.3)	74.2 CH	3.57 (1H, dd, 9.6, 3.2)	74.7 CH
4″	3.92 (1H, d, 3.3)	69.4 CH	3.92 (1H, d, 3.2)	70.1 CH
5″	3.68 (1H, t, 7.2)	76.3 CH	3.63 (1H, m)	77.0 CH
6″	H_a 3.77 (1H, m)	61.8 CH_2	H_a 3.77 (1H, m)	62.3 CH_2
	H_b 3.76 (1H, m)		H_b 3.76 (1H, m)	

^{1}H- and ^{13}C-NMR spectral data were measured at 500 and 125 MHz, respectively, in $CDCl_3$. The assignments were aided by ^{1}H–^{1}H COSY, DEPT, HMQC and HMBC.

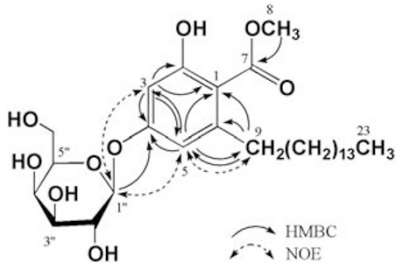

Figure 2 Key HMBC and NOE correlations of **1**.

inhibition against the enoyl-ACP reductases, aquastatin C did not prevent bacterial growth of *S. aureus*, methicillin-resistant *S. aureus* and *S. pneumonia*. To more clearly examine the structure-activity relationship, effects of 2-hydroxy-6-methyl-benzoic acid (**4**) and 2,4-dihydroxy-5-pentadecyl-benzoic acid methyl ester (**5**) against enoyl-ACP reductases and bacterial growth was investigated. Compound **5** was obtained from acid hydrolysis of **2**. As 5-methyl-benzene-1,3-diol (**6**) was made instead of 2,4-dihydroxy-6-methyl-benzoic acid from the acid hydrolysis, **4** was purchased (Johnson Matthey Company, UK). Interestingly, all of **4**–**6** did not inhibited *S. aureus* FabI as well as *S. pneumoniae* FabK even at 100 μM (Table 3). These compounds also did not show antibacterial activity against *S. aureus*, methicillin-resistant *S. aureus* and *S. pneumonia*. These results clearly indicated that both 2-hydroxy-6-methyl-benzoic acid and 2,4-dihydroxy-5-pentadecyl-benzoic acid are important for its anti-enoyl-ACP reductase activity as well as antibacterial activity.

Table 3 Comparison of inhibitory activity of compounds 1–3 and related compounds against enoyl-ACP reductases and bacterial viability

	$IC_{50}(\mu M)$			$MIC\ (\mu g\,ml^{-1})$			
	saFabI	*spFabK*	*mtInhA*	*S. aureus*	*MRSA*	*S. pneumoniae*	*M. tuberculosis*
1	>100	>100	>100	>128	>128	>128	NT
2	3.2	9.2	9.6	32	16	128	>128[a]
3	3.4	10.6	18.4	32	32	64	NT
4	>100	>100	>100	>128	>128	>128	NT
5	>100	>100	>100	>128	>128	>128	NT
6	>100	>100	>100	>128	>128	>128	NT

Abbreviations: ACP, acyl carrier protein; *M. tuberculosis*, *Mycobacterium tuberculosis* H37Rv; MRSA, methicillin-resistant *Staphylococcus aureus* CCARM 3167; mtInhA, *M. tuberculosis* InhA; NT, not tested; saFabI, *S. aureus* FabI; *S. aureu, S. aureus* RN4220; spFabK, *Streptococcus pneumonia* FabK, *S. pneumonia* KCTC 5412.
[a]Compound **2** inhibited the growth of *M. tuberculosis* by 17.9% at 128 $\mu g\,ml^{-1}$.

As InhA is the FabI homolog from *Mycobacterium tuberculosis*, effect of aquastatins A–C, and related compounds on *M. tuberculosis* InhA was examined. Aquastatins A and B inhibited *M. tuberculosis* InhA with IC_{50} of 9.6 and 18.4 μM, respectively, whereas aquastatin C and other compounds did not affect at 100 μM. Aquastatin A showed weak anti-bacterial activity against *M. tuberculosis* with

inhibiting bacterial growth of *M. tuberculosis* by 17.9% at 128 μg ml^{-1}. The weak antibacterial activity of aquastatin A against *M. tuberculosis* may be because of permeability barrier, as *M. tuberculosis* contains a thick cell wall compared with *S. aureus*.

In summary, aquastatin C is a rare metabolite incorporating one aromatic ring with a sugar and an aliphatic chain, which was isolated from liquid fermentation cultures of *Sporothrix* sp. FN611. Aquastatin C is a new glycoaromatic derivative of aquastatin A in which the 2-hydroxy-6-methyl benzoic acid ring of aquastatin A was deleted. Aquastatin C completely lost inhibitory activity of aquastatin A against bacterial enoyl-ACP reductases, such as *S. aureus* FabI, *S. pneumoniae* FabK and *M. tuberculosis* InhA. Also aquastatin C did not inhibited bacterial growth of *S. aureus*, methicillin-resistant *S. aureus* and *S. pneumonia*. The 2-hydroxy-6-methyl benzoic acid, the other moiety of aquastatin A, as well as the 2,4-dihydroxy-5-pentadecyl-benzoic acid methyl ester also did not show the FabI-inhibitory and antibacterial activity, indicating that both moieties are necessary for its inhibitory activity against enoyl-ACP reductases and bacterial growth. These results could provide new insights into the development of a selective mechanism-based antibacterial agent with targeting FabI.

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