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ORIGINAL ARTICLE

Haplofungins, new inositol phosphorylceramide synthase inhibitors, from *Lauriomyces bellulus* SANK 26899 II. Structure elucidation

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Eight new inositol phosphorylceramide synthase inhibitors: haplofungin A, B, C, D, E, F, G and H, were discovered in a culture broth of the fungus *Lauriomyces bellulus* SANK 26899. The planar structures for these haplofungins were elucidated by various spectroscopic analyses and a GC/MS analysis of their degradation products. All eight compounds were found to comprise an arabinonic acid moiety linked through an ester bond to a modified long alkyl chain.
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INTRODUCTION

In our screening program for new inositol phosphorylceramide (IPC) synthase inhibitors, we discovered eight new compounds named haplofungin A (**1**), B (**2**), C (**3**), D (**4**), E (**5**), F (**6**), G (**7**) and H (**8**) from a culture broth of *Lauriomyces bellulus* SANK 26899 (Figure 1). In the preceding paper,[1] we described the taxonomy of the producing strain and the production, isolation and biological activities of these haplofungins. Here, we report the physicochemical properties and structure elucidation of these haplofungins.

RESULTS

Physicochemical properties

The physicochemical properties of **1–8** are summarized in Table 1. These compounds were soluble in methanol, ethyl acetate and dimethylsulfoxide, but were insoluble in water and *n*-hexane. The IR spectra of these compounds showed characteristic absorption bands for hydroxyl (3400 cm^{-1}), carbonyl and carboxyl groups (1740–1661 cm^{-1}). The similarity of the UV spectra (λ_{max} 236–238 nm) of these compounds indicated that they have the same α,β-unsaturated carbonyl system. ^{1}H and ^{13}C NMR data of haplofungins are summarized in Tables 2 and 3, respectively.

Structure elucidation of haplofungins

Structure of 1. The structure of **1** was established on the basis of the NMR and MS data. The molecular formula of **1** was determined to be $C_{33}H_{60}O_9$ based on high-resolution time-of-flight mass spectrometry (HRTOF-MS), which gave an $(M+Na)^+$ ion at *m/z* 623.4122 (calculated for $C_{33}H_{60}O_9Na$, 623.4135). The ^{13}C NMR spectra indicated that **1** contains five methyls, 15 methylenes, one oxygenated methylene, three methines, four oxygenated methines, one sp^2 methine, one sp^2 quaternary carbon, one carbonyl carbon, one ester carbonyl carbon and one carboxyl carbon. To fulfill the molecular formula of **1**, the presence of four hydroxyl groups was suggested. The presence of a long alkyl chain was readily inferred from a broad equivalent signal resonating from δ 1.26 to δ 1.32, which was observed in the ^{1}H NMR spectrum. The connectivity of protons and carbons was established by the heteronuclear single quantum coherence (HSQC) spectrum. Double quantum filtered correlation spectroscopy (DQF COSY) and heteronuclear single quantum coherence homonuclear Hartmann–Hahn spectroscopy (HSQC-HOHAHA) experiments showed the three proton spin systems drawn in bold lines (Figure 2). The first proton spin system was found to be a long alkyl chain moiety from the H-5 olefinic methine through the H-13-oxygenated methine to the terminal methyl at H-24. Two blanched methyl groups (C-27, C-28) connected to the C-6 and C-8 methine carbons at δ 33.0 and δ 32.1 were also included in this system. The second proton spin system consisted of an H-2 methine adjacent to an H-25 methyl. The connectivities of three oxygenated methines (H-2′, H-3′ and H-4′) and one oxygenated methylene (C-5′) were established as the third spin system.

A heteronuclear multiple bond correlation (HMBC) experiment was used to confirm the assignments of above proton spin systems and establish the connectivity between the partial structures constructed above (Figure 2). The H-5 allylic methine proton at δ 6.64, in the first spin system, was assigned at the β-position of α,β-unsaturated carbonyl system by the observation of ^{1}H–^{13}C long-range correlations

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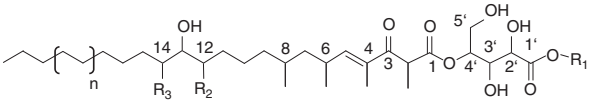
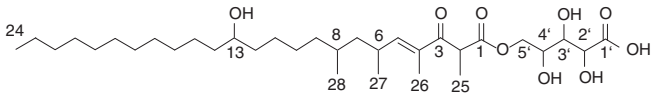
from the H-5 olefinic proton to C-3 keto carbonyl and C-4 quaternary sp^2 carbon. The correlations with the H-26 methyl group at δ 1.80 to C-3, C-4 and C-5 confirmed the assignment of the methyl to be directly attached at C-4. The correlations with the H-25 methyl group to C-2, C-1 ester carbonyl carbon and C-3 keto carbonyl carbon and from the H-2 methine proton at δ 4.47 to C-1, C-3 and C-4 confirmed a 1,3-dicarbonyl-2-methyl system. The configuration of the Δ^4 double bond was determined to be *E* by NOEs observed between the H-26 methyl protons and the other two methyl protons at H-25 and H-27. The presence of an aldonate moiety in **1** was shown by the observation of the ^1H–^{13}C long-range coupling from oxygenated H-2′ methine proton at δ 4.05 to C-1′ carboxyl carbon in the third spin system. As the long-range correlation with the H-4′-oxygenated methine proton at δ 4.97 to C-1 ester carbonyl carbon was observed in the HMBC experiment, it was shown that the aldonate moiety was connected to the 1,3-dicarbonyl system through an ester bond between C-1 and C-4′.



Haplofungin	R_1	R_2	R_3	n	M.W.
A (**1**)	H	H	H	2	600
C (**3**)	CH_3	H	H	2	614
D (**4**)	H	H	H	1	572
E (**5**)	H	H	H	3	628
F (**6**)	H	H	OH	2	616
G (**7**)	CH_3	H	H	1	586
H (**8**)	H	OH	H	2	616



Haplofungin B (**2, M**.W. 600)

Figure 1 Structures of haplofungins.

To identify the aldonate, **1** was treated with 5% HCl/MeOH and then with *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA). The reaction mixture was applied to GC/MS directly. The TMS derivative of the aldonate moiety was detected as a peak with a retention time of 12.56 min, which was identical to that of the authentic arabinono-1,4-lactone derivative.

It was thought that it would be possible to determine the length of the alkyl chain and the substituted position of the hydroxyl group by analyzing the EI-MS fragmentation of the keto analog obtained through the decarboxylation at C-1 of the fatty-acid chain because the β-keto acid was unstable under acidic or alkaline conditions when

Table 1 Physicochemical properties of haplofungins

	1	*2*	*3*	*4*
Appearance	*White powder*	*White powder*	*White powder*	*White powder*
Molecular weight	600	600	614	572
Molecular formula	$C_{33}H_{60}O_9$	$C_{33}H_{60}O_9$	$C_{34}H_{62}O_9$	$C_{31}H_{56}O_9$
HR-MS (*m/z*)				
Calculated:	623.4135	623.4135	637.4291	595.3822
Found:	623.4122 (M+Na)+ [a]	623.4134 (M+Na)+ [a]	637.4292 (M+Na)+ [a]	595.3830 (M+Na)+ [b]
UV λ_{max} (methanol) nm (ε)	238 (5,970)	236 (8,588)	236 (7,970)	236 (10,800)
IR ν_{max} (KBr) cm^{-1}	3407, 3137, 2928, 2856, 1737, 1679, 1449, 1381, 1316, 1213, 1146, 1037, 845, 803, 726	3353, 2955, 2919, 2850, 1785, 1737, 1666, 1465, 1456, 1377, 1317, 1283, 1251, 1201, 1131, 1093, 1052	428, 2953, 2925, 2854, 2729, 1744, 1663, 1456, 1376, 1251, 1216, 1130, 1073, 1036, 951	3408, 2926, 2855, 1740, 1663, 1457, 1377, 1312, 1249, 1201, 1129, 1073, 1040, 968
$[\alpha]_{24}^{D}$ (methanol)	−20.0° (*c* 2.0)	−33.0° (*c* 1.0)	−58.0° (*c* 1.1)	−38.0° (*c* 1.0)
HPLC retention time (min)[c]	15.2	15.6	17.4	13.5
	5	*6*	*7*	*8*
Appearance	*White powder*	*White powder*	*White powder*	*White powder*
Molecular weight	628	616	586	616
Molecular formula	$C_{35}H_{64}O_9$	$C_{33}H_{60}O_{10}$	$C_{32}H_{58}O_9$	$C_{33}H_{60}O_{10}$
HR-MS (*m/z*)				
Calculated:	651.4448	639.4084	609.3978	661.3903
Found:	651.4479 (M+Na)+ [a]	639.4105 (M+Na)+ [b]	609.4000 (M+Na)+ [b]	661.3920 (M+2Na-H)+ [b]
UV λ_{max} (methanol) nm (ε)	237 (20,000)	237 (12,000)	236 (12,500)	236 (16,300)
IR ν_{max} (KBr) cm^{-1}	3378, 2956, 2920, 2851, 1739, 1665, 1466, 1457, 1377, 1249, 1215, 1131, 1074, 1040, 967	3372, 2924, 2854, 1738, 1661, 1457, 1377, 1314, 1245, 1215, 1127, 1073, 1038, 967	3394, 2955, 2928, 2855, 1743, 1161, 1456, 1377, 1216, 1130, 1074, 1037, 952	3336, 2954, 2922, 2872, 2853, 1737, 1664, 1458, 1377, 1247, 1213, 1127, 1069, 1042, 947
$[\alpha]_{24}^{D}$ (methanol)	−28.0° (*c* 0.1)	−50.0° (c 0.1)	−50.0° (*c* 0.1)	−32.0° (*c* 0.1)
HPLC retention time (min)[c]	18.2	11.9	10.6	11.2

[a]LC/ESI-TOF MS, liquid chromatography coupled with time of flight mass spectrometry using electrospray ionization.
[b]HRFAB MS, high-resolution fast atom bombardment mass spectrscopy
[c]Condition of HPLC analysis is described in experimental section.

Table 2 ^{1}H NMR spectral data of haplofungins

	1		*2*		*3*		*4*		*5*		*6*		*7*		*8*	
Carbon no.	δ_H[a]	*mult., J=Hz*	δ_H[b]	*mult., J=Hz*	δ_H[c]	*mult., J=Hz*	δ_H[a]	*mult., J=Hz*	δ_H[a]	*mult., J=Hz*	δ_H[a]	*mult., J=Hz*	δ_H[a]	*mult., J=Hz*	δ_H[a]	*mult., J=Hz*
2	4.47	1H, d, 7.1	4.56	1H, d, 6.9	4.42	1H, d, 7.0	4.47	1H, d, 7.1	4.47	1H, d, 7.1	4.47	1H, d, 7.1	4.47	1H, d, 7.1	4.47	1H, d, 7.1
5	6.64	1H, dd, 1.1, 9.3	6.77	1H, d, 9.4	6.61	1H, dd, 1.1, 9.3	6.64	1H, d, 9.3	6.64	1H, d, 9.6	6.64	1H, d, 9.5	6.64	1H, d, 9.6 Hz	6.64	1H, d, 9.3
6	2.77	1H, m	2.70	1H, m	2.64	1H, m	2.75	1H, m	2.76	1H, m	2.76	1H, m	2.78	1H, m	2.77	1H, m
7	1.32	2H, m	1.22	1H, m	1.17	1H, m	1.33	1H, m	1.34	2H, m	1.25	2H, m	1.25	2H, m	1.32	2H, m
			1.34	1H, m	1.31	1H, m	1.40	1H, m								
8	1.65	1H, m	1.44	1H, m	1.35	1H, m	1.45	1H, m	1.45	1H, m	1.65	1H, m	1.42	1H, m	1.65	1H, m
9	1.12	2H, m	1.41	2H, m	1.03	1H, m	1.14	1H, m	1.15	1H, m	1.12	2H, m	1.12	1H, m	1.12	2H, m
					1.17	1H, m	1.39	1H, m	1.40	1H, m						
10	1.29	2H, m	1.46	2H, m	1.29	2H, m	1.30	2H, m	1.30	2H, m	1.29	2H, m	1.29	2H, m	1.29	2H, m
11	1.29	2H, m	1.14	1H, m	1.29	2H, m	1.30	2H, m	1.30	2H, m	1.29	2H, m	1.29	2H, m	1.58	2H, m
			1.62	1H, m												
12	1.42	2H, m	1.69	2H, m	1.23	2H, m	1.42	2H, m	1.42	2H, m	1.54	2H, m	1.37	2H, m	3.29	1H, m
13	3.49	1H, m	3.90	1H, m	3.40	1H, m	3.45	1H, m	3.48	1H, m	3.33	1H, m	3.49	1H, m	3.29	1H, m
14	1.44	2H, m	1.68	2H, m	1.29	2H, m	1.42	2H, m	1.42	2H, m	3.33	1H, m	1.37	2H, m	1.58	2H, m
15	1.29	2H, m	1.74	2H, m	1.29	2H, m	1.30	2H, m	1.29	2H, m	1.54	2H, m	1.29	2H, m	1.30	2H, m
16	1.29	2H, m	1.29	2H, m	1.29	2H, m	1.30	2H, m	1.29	2H, m	1.30	2H, m	1.29	2H, m	1.30	2H, m
17	1.29	2H, m	1.29	2H, m	1.29	2H, m	1.30	2H, m	1.29	2H, m	1.30	2H, m	1.29	2H, m	1.30	2H, m
18	1.29	2H, m	1.29	2H, m	1.29	2H, m	1.30	2H, m	1.29	2H, m	1.30	2H, m	1.29	2H, m	1.30	2H, m
19	1.29	2H, m	1.29	2H, m	1.29	2H, m	1.30	2H, m	1.29	2H, m	1.30	2H, m	1.29	2H, m	1.30	2H, m
20	1.29	2H, m	1.29	2H, m	1.29	2H, m	1.30	2H, m	1.29	2H, m	1.30	2H, m	1.29	2H, m	1.30	2H, m
21	1.29	2H, m	1.29	2H, m	1.29	2H, m	1.33	2H, m	1.29	2H, m	1.30	2H, m	1.33	2H, m	1.30	2H, m
22	1.29	2H, m	1.25	2H, m	1.29	2H, m	0.90	3H, m	1.29	2H, m	1.30	2H, m	0.89	3H, m	1.30	2H, m
23	1.33	2H, m	1.27	2H, m	1.33	2H, m	1.30	3H, d, 4.5	1.29	2H, m	1.41	2H, m	1.31	3H, d, 7.1	1.41	2H, m
24	0.89	3H, m	0.88	3H, m	0.89	3H, m	1.80	3H, m	1.29	2H, m	0.89	3H, m	1.80	3H, brs	0.90	3H, m
25	1.31	3H, m	1.51	3H, d, 6.9	1.32	3H, m	1.05	3H, d, 6.7	1.34	2H, m	1.31	3H, d, 7.1	1.06	3H, d, 6.6	1.31	3H, d, 7.0
26	1.80	3H, d, 1.1	1.92	3H, brs	1.80	3H, d, 1.1	0.91	3H, m	0.89	3H, m	1.80	3H, d, 7.2	0.91	3H, d, 6.3	1.81	3H, brs
27	1.06	3H, d, 6.6	1.03	3H, d, 6.3	1.06	3H, d, 6.6			1.30	3H, d, 7.1	1.05	3H, d, 6.0			1.04	3H, d, 6.6
28	0.92	3H, m	0.90	3H, m	0.92	3H, m			1.80	3H, brs	0.91	3H, d, 7.0			0.91	3H, d, 6.3
29									1.31	3H, d, 6.7						
30									0.90	3H, m						
2′	4.05	1H, d, 2.3	5.46	1H, brs	4.05	1H, d, 2.3	4.07	1H, d, 2.3	4.05	1H, d, 2.3	4.06	1H, d, 2.3	4.11	1H, d, 1.3	4.05	1H, d, 2.3
3′	4.09	1H, dd, 2.5, 9.3	4.82	1H, m	4.09	1H, dd, 2.3, 9.3	4.11	1H, dd, 2.3, 9.3	4.09	1H, dd, 2.3, 9.3	4.08	1H, dd, 2.3, 9.5	4.06	1H, dd, 1.3, 9.3	4.09	1H, dd, 2.3, 9.5
4′	4.97	1H, ddd, 1.5, 4.5, 9.3	4.83	1H, m	4.97	1H, ddd, 1.9, 9.3, 4.5	4.98	1H, ddd, 1.9, 4.5, 9.3	4.97	1H, ddd, 1.9, 4.5, 12.1	4.97	1H, ddd, 2.5, 4.6, 9.5	4.98	1H, ddd, 2.6, 4.5, 9.3	4.97	1H, ddd, 1.9, 4.5, 9.3
5′	3.76	1H, dd, 1.9, 12.1	4.94	1H, dd, 5.1, 10.9	3.76	1H, dd, 1.9, 12.1	3.78	1H, dd, 4.5, 12.1	3.76	1H, dd, 1.9, 12.1	3.76	1H, dd, 4.6 , 12.3	3.77	1H, dd, 2.6, 12.3	3.76	1H, dd, 1.9, 12.1
	3.88	1H, dd, 4.5, 12.1	5.12	1H, dd, 1.9, 10.9	3.88	1H, dd, 4.5, 12.1	3.89	1H, dd, 1.9, 12.1	3.88	1H, dd, 4.5, 12.1	3.88	1H, dd, 2.5, 12.3	3.87	1H, dd, 4.5, 12.3	3.88	1H, dd, 4.5, 12.1
5′-OCH_3					3.63	3H, s							3.75	3H, s		

[a]Chemical shifts are shown with reference to methanol-d_4 as δ_H 3.31.
[b]Chemical shifts are shown with reference to pyridine-d_5 as δ_H 8.74.
[c]Chemical shifts are shown with reference to dimethylsulfoxide-d_6 as δ_H 2.50.

Table 3 ^{13}C NMR spectral data of haplofungins

	1		*2*		*3*		*4*		*5*		*6*		*7*		*8*	
Carbon no.	δ_C[a]	*mult.*	δ_C[b]	*mult.*	δ_C[c]	*mult.*	δ_C[a]	*mult.*	δ_C[a]	*mult.*	δ_C[a]	*mult.*	δ_C[a]	*mult.*	δ_C[a]	*mult.*
1	172.1	s	172.8	s	170.2	s	172.7	s	172.7	s	172.6	s	175.7	s	172.0	s
2	47.6	d	49.9	d	45.8	d	48.2	d	48.4	d	47.7	d	48.2	d	47.6	d
3	200.9	s	198.6	s	197.8	s	201.6	s	201.5	s	201.0	s	201.6	s	200.9	s
4	135.5	s	135.1	s	133.4	s	136.2	s	136.5	s	135.9	s	136.1	s	135.5	s
5	153.3	d	151.5	d	150.9	d	153.9	d	153.9	d	153.3	d	153.9	d	153.3	d
6	33.0	d	32.2	d	31.0	d	33.6	d	33.6	d	33.1	d	33.6	d	33.0	d
7	45.6	t	44.9	t	43.6	t	46.2	t	46.2	t	45.6	t	46.2	t	45.5	t
8	32.1	d	31.4	d	30.1	d	32.7	d	32.7	d	32.2	d	32.7	d	32.1	d
9	38.0	t	37.7	t	36.2	t	38.0	t	38.0	t	38.1	t	38.9	t	38.2	t
10	28.3	t	28.0	t	26.5	t	29.0	t	28.9	t	28.4	t	28.9	t	23.9	t
11	26.9	t	27.0	t	25.6	t	27.9	t	27.9	t	27.2	t	27.9	t	34.1	t
12	38.8	t	39.0	t	37.2	t	39.2	t	39.2	t	33.8	t	39.2	t	76.1	d
13	75.6	d	71.5	d	69.5	d	73.2	d	73.2	d	76.2	d	73.2	d	76.2	d
14	38.6	t	39.1	t	37.2	t	39.1	t	39.2	t	76.2	d	39.1	t	33.8	t
15	27.2	t	27.3	t	25.2	t	27.6	t	27.6	t	33.7	t	27.6	t	27.2	t
16	30.6	t	30.1	t	28.8	t	31.3	t	31.3	t	24.0	t	31.3	t	30.5	t
17	31.0	t	30.4	t	29.2	t	31.7	t	31.5	t	30.9	t	31.5	t	30.9	t
18	30.9	t	30.6	t	29.1	t	31.6	t	31.5	t	30.9	t	31.6	t	30.9	t
19	30.9	t	30.5	t	29.1	t	31.5	t	31.5	t	30.9	t	31.7	t	30.9	t
20	30.9	t	30.5	t	29.1	t	33.9	t	31.5	t	30.9	t	33.9	t	30.9	t
21	30.9	t	30.8	t	29.1	t	24.6	t	31.5	t	30.9	t	24.6	t	30.9	t
22	33.2	t	32.6	t	31.3	t	15.3	q	31.5	t	33.3	t	12.6	q	33.2	t
23	23.9	t	23.4	t	22.1	t	15.6	q	31.5	t	26.4	t	15.6	q	24.5	t
24	14.6	q	14.8	q	14.1	q	15.2	q	33.9	t	14.7	q	15.3	q	14.6	q
25	15.0	q	15.2	q	14.0	q	20.8	q	24.6	t	15.0	q	20.9	q	15.0	q
26	12.0	q	12.5	q	11.3	q	21.2	q	15.3	q	12.0	q	21.2	q	12.0	q
27	20.1	q	20.3	q	19.3	q	—		15.6	q	20.2	q	—		20.1	q
28	20.6	q	20.6	q	19.9	q	—		12.7	q	20.6	q	—		20.6	q
29	—		—		—		—		20.8	q	—		—		—	
30	—		—		—		—		21.2	q	—		—		—	
1′	176.7	s	178.0	s	173.3	s	177.4	s	177.1	s	176.6	s	172.6	s	176.5	s
2′	71.6	d	72.4	d	70.4	d	72.1	d	72.2	d	71.6	d	72.5	d	71.5	d
3′	71.4	d	74.5	d	69.4	d	72.0	d	72.0	d	71.1	d	72.0	d	71.3	d
4′	75.5	d	70.3	d	73.7	d	76.1	d	76.1	d	75.5	d	75.8	d	75.4	d
5′	61.7	t	69.3	t	59.3	t	62.3	t	62.4	t	61.7	t	62.3	t	61.7	t
5′-OCH3	—		—		51.6	q	—		—		—		49.3	q	—	

[a]Chemical shifts are shown with reference to methanol-d_4 as δ_C 49.0.
[b]Chemical shifts are shown with reference to pyridine-d_6 as δ_C 150.4.
[c]Chemical shifts are shown with reference to dimethylsulfoxide-d_6 as δ_C 39.5.

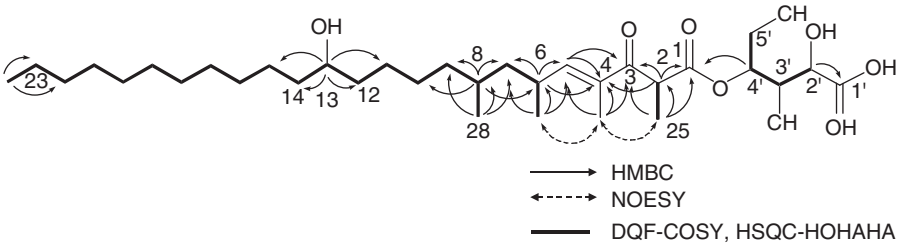


Figure 2 Selected ^{1}H–^{1}H COSY, HMBC and NOE correlations in haplofungin A (**1**).

making hydorolysate of haplofungins. For this purpose, **1** was treated with 5% HCl/MeOH at 105°C for 10 h. The resultant was concentrated *in vacuo* followed by treatment with BSTFA in pyridine for 5 min. Their EI-MS fragment patterns and retention times in the GC/MS analysis are shown in Figure 3 and Table 4. The substituted position of the hydroxyl group was determined by the alkyl chain length from the α-cleavage sites at each side of the hydroxyl group. Fragmentation of the alkyl chain derivative (**1a**) from **1** on EI-MS gave fragments at *m/z* 257 and 325, indicating that the oxidation site is C-13 (Figure 3). From these results, the planar structure of **1** was established as shown in Figure 1.

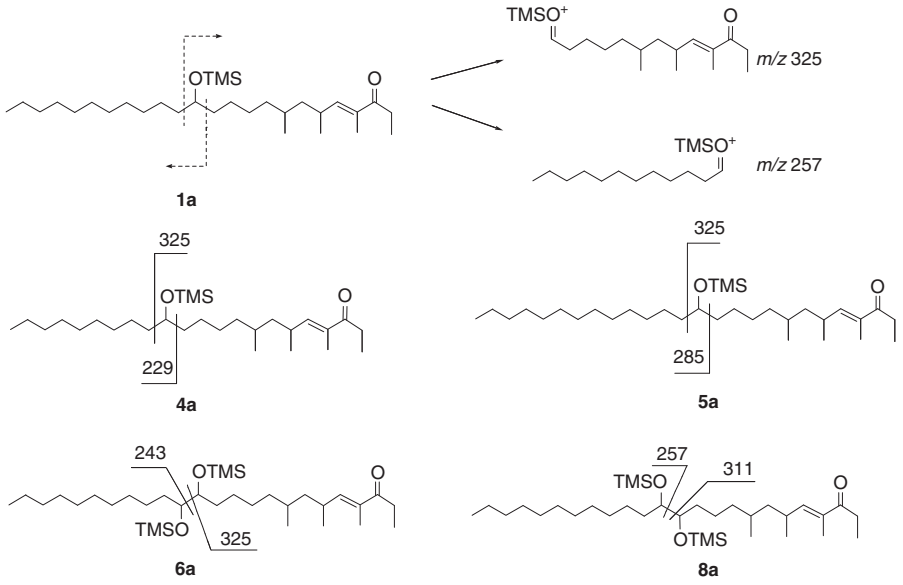


Figure 3 EI-MS fragmentation patterns of derived alkyl chain moieties. The ketone and long-chain moieties were produced by α-cleavage reactions that occur on either side of the hydroxyl group with loss of enol ether moiety. For example, fragmentation of **1a** on EI-MS gave fragments at *m/z* 357 and 325, indicating that the oxidation site is C-13.

Table 4 GC-MS retention times and EI-MS fragmentation patterns of TMS derivatives

	t_R (min)				
Compound	*Arabinono lactone*	*Alkyl chain*	*Derivative*	*EI-MS fragment ions (m/z)*	
1	12.56	33.91	**1a**	257	325
3	12.62	33.96	**1a**	257	325
4	12.58	31.23	**4a**	229	325
5	12.63	36.52	**5a**	285	325
6	12.55	35.52	**6a**	243	325
7	12.62	31.26	**4a**	229	325
8	12.54	35.39	**8a**	257	311

Structure elucidation of the minor components

As described in the preceding paper,[1] compounds **2–8** were isolated as minor components from a culture broth of SANK 26899. The analysis of each compound by LC/MS afforded molecular weights ranging from 572 Da to 628 Da. Their mass differences were easily explained as the number of CH_2 units or hydroxyl substituents from **1**. To determine the location of hydroxyl groups, number of aliphatic carbons, and to identify an aldonic acid moiety, GC/MS analysis of TMS derivatives prepared from the methanolysis products of all minor compounds were used.

In addition to the results shown in Figure 3 and Table 4, the structures of the minor components were finally determined by 1D and 2D NMR spectra, as follows:

*Structure of haplofungin B (**2**).* The molecular formula of **2** was determined as $C_{33}H_{60}O_9$ by HRTOF-MS, which was the same as that of **1**. The ^1H and ^{13}C NMR spectra of **2** were very similar to those of **1**, except for the signals for the aldonic acid moiety. In the HMBC experiment, ^1H–^{13}C long-range correlations were observed from H-5′a- and 5′b-oxygenated methylene protons at δ 4.94 and 5.12 to the C-1 ester carbonyl carbon at δ 172.8, which established that the C-5′ in aldonate moiety is attached to the C-1 in the fatty-acid moiety (Figure 1). During purification, it was observed that **1** was easily converted to **2** under alkaline conditions (data not shown). The structure of **2** was thus determined to be an acyl rearrangement product of **1**.

*Structure of haplofungin C (**3**).* The molecular formula of **3**,determined as $C_{34}H_{62}O_9$ by HRTOF-MS, differed from that of **1** by one CH_2 unit. The ^1H and ^{13}C NMR spectra in DMSO-d_6 indicated the presence of methyl ester (δ_H 3.63, δ_C 51.6). The structures of the aldonate moiety and the fatty-acid moiety were identified by GC/MS analysis (Table 4). The fragment patterns of the hydrolyzed TMS derivatives of the methanolysis products were identical with those of **1**. In the HMBC spectrum, correlation from 1′-OCH_3 methoxyl protons at δ 3.63 to C-1′ carbonyl carbon at δ 173.3 in the aldonic acid moiety was observed. A ^1H–^{13}C long-range correlation was also observed from H-4′-oxygenated methine proton at δ 4.97 to C-1 ester carbonyl carbon at δ 170.2, which established that the C-4′ in aldonate moiety is attached to the C-1 in the fatty acid moiety. From these results, the structure of **3** was elucidated to be the methyl ester of **1** (Figure 1).

*Structure of haplofungin D (**4**).* The molecular formula of **4** was determined as $C_{31}H_{56}O_9$ by high-resolution fast atom bombardment mass spectrscopy (HR-FABMS), which was different from that of **1** by two CH_2 units. The structures of the aldonate moiety and the fatty-acid moiety were also determined by GC/MS analysis and the fragment ion (*m/z* 229) originated from the TMS derivative (**4a**) of the fatty acid indicated that the length of the alkyl chain was shorter than **1** by two CH_2 units (Figure 3, Table 4). The ^{13}C NMR spectrum readily showed the absence of two methylene units in the alkyl chain. In the HMBC spectrum, ^1H–^{13}C long-range correlation was observed from oxygenated H-4′ methine proton at 4.98 to C-1 ester carbonyl carbon at 172.7, which established that the C-4′ in aldonic acid moiety is attached to the C-1 in the fatty-acid moiety. Combining the results of the NMR and GC/MS experiments, the structure of **4** was determined to be as shown in Figure 1.

*Structure of haplofungin E (**5**).* The molecular formula of **5** was determined as $C_{35}H_{64}O_9$ by HRTOF-MS and it was shown that **5** was two CH_2 units larger than **1**. The identification of the aldonate moiety and the structure analysis of the fatty-acid moiety were carried out by GC/MS analysis. The fragment ion (*m/z* 285) from the TMS derivative (**5a**) of the fatty acid indicated that the alkyl chain length was longer than that of **1** by two CH_2 units (Figure 3, Table 4). Two newly appeared methylene carbons, in the alkyl chain, were observed in the ^{13}C NMR spectrum. In the HMBC spectrum, ^{13}C–^{1}H long-range correlation from the oxygenated methine proton at δ 4.97 (H-4′) to the ester carbonyl carbon at δ 172.7 (C-1) indicated that the C-4′ in the aldonate moiety is attached to ester carbonyl carbon (C-1) in the fatty-acid moiety. From these results, the structure of **5** was determined to be as shown in Figure 1.

*Structure of haplofungin F (**6**).* The molecular formula of **6** was determined to be $C_{33}H_{60}O_{10}$ by HR-FABMS and shown to have one additional oxygen in the molecule compared to **1**. The ^{13}C NMR spectrum readily showed the presence of one newly observed oxygenated methine carbon signal at δ 76.2. These data strongly suggested that one hydroxyl group was introduced into the structure of **1**. To clarify the oxygenated sites, TMS derivatives of the aldonate moiety and fatty-acid moiety were subjected to GC/MS analysis and the appearance of the fragment ions (*m/z* 243 and 325) caused by the oxidative cleavage of 1,2-diol in the TMS derivative of the fatty acid (**6a**) showed the substituted positions of two hydroxyl groups to be at C-13 and C-14 (Figure 3, Table 4). In the HMBC spectrum, ^{1}H–^{13}C long-range correlation was observed from H-4′-oxygenated methine proton at δ 4.97 to C-1 ester carbonyl carbon at δ 172.6, which established that the C-4′ in aldonic acid moiety is attached to C-1 in the fatty-acid moiety. From these results, the structure of **6** was determined to be as shown in Figure 1.

*Structure of haplofungin G (**7**).* The molecular formula of **7** was determined to be $C_{32}H_{58}O_9$ by HR-FABMS. It differs from that of **4** by one CH_2 unit. The ^{1}H and ^{13}C NMR spectra indicated the presence of a methoxyl group (δ_H 3.75, δ_C 49.3). GC/MS analysis of the TMS derivative showed the same fragment pattern as that of **4** (Figure 3, Table 4). In the HMBC spectrum, correlation between 1′-OCH_3 protons at δ 3.75 to C-1′ carbonyl carbon at δ 172.6 in the aldonic acid moiety was observed. ^{1}H–^{13}C long-range correlation was also observed from the H-4′ oxygenated methine proton at δ 4.98 to the C-1 ester carbonyl carbon at δ 175.7, which established that the C-4′ in the aldonic acid moiety is attached to C-1 in the fatty-acid moiety (Figure 1). From these results, the structure of **7** was established to be the methyl ester of **4**.

*Structure of haplofungin H (**8**).* The molecular formula of **8** was determined to be $C_{33}H_{60}O_{10}$ by HR-FABMS, which was the same as **6**. Typical two fragment ions (m/z 311 and 257) derived from the TMS derivative of the fatty acid (**8a**) by the 1,2-diol cleavage between C-12 and C-13 were observed. The ^{13}C NMR spectrum readily showed the presence of one newly observed oxygenated methine carbon signal at 76.1 in the alkyl chain. As the same hydroxylation sites as **6** were expected in this case, GC/MS analysis was carried out. Consequently, the substituted positions of the two hydroxyl groups were determined to be at C-12 and 13 (Figure 3, Table 4). ^{1}H–^{13}C long-range correlation was observed from the oxygenated H-4′ methine proton at δ 4.97 to the C-1 ester carbonyl carbon at δ 172.0, which established that the C-4′ in the aldonic acid moiety is attached to the C-1 in the fatty-acid moiety. Thus, the structure of **8** was determined to be the C-12-hydroxylated homolog of **1** as shown in Figure 1.

DISCUSSION

Eight new compounds were isolated as fungal IPC synthase inhibitors. Their planer structures have been elucidated by 1D and 2D NMR experiments and GC/MS analysis of the TMS derivatives from degradation products prepared by methanolysis. From these results, it was shown that these homologs were composed of an arabinonic acid moiety and a β-keto acid with a long unsaturated hydroxyalkyl chain as partial structures. Interestingly, these units have a high similarity to the partial structures of two known IPC synthase inhibitors, khafrefungin[3] and rustmicin.[4,5] The arabinonic acid and the hydroxylated long-chain fatty-acid units are common to khafrefungin and the α-methyl-β-keto acid unit is common to rustmicin. These units are reported to be indispensable for the biological activities of these compounds.[6–8] A 14-membered macrolide, the same as rustmicin, has been obtained by macrolactonization between C-1 and C-13 of β-keto acid prepared from **1** by hydrolysis under mild alkaline condition. However, this macrolide showed no biological activity (data not shown). It was evident that haplofungin has a very unique structure which possesses partial structures of these two known compounds. The core structure of haplofungins also seems to mimic the structure of phytoceramide, which is the substrate of IPC synthase. However, their antifungal activities were weak,[1] in contrast to the strong inhibitory activities against IPC synthase. If the structural features and the antifungal activities of haplofungins are improved, these derivatives will be candidates for new chemotherapeutic drugs for fungal diseases. The absolute structure of haplofungin A will be reported in the succeeding paper.[9]

EXPERIMENTAL SECTION

General experimental procedures

The 1D and 2D NMR spectra of haplofungins were recorded on a Bruker AVANCE 500 spectrometer (Bruker, Rheinstetten, Germany) equipped with a cryogenic probe. The data set acquired for each sample consisted of 1D ^{1}H, ^{13}C NMR, gs-DQF-COSY, NOESY, gs-HSQC, gs-HMBC and gs-HSQC-HOHAHA experiments which were adopted from Bruker standard pulse microprograms. The HR-FABMS spectra were recorded on a Micromass Autospec mass spectrometer (Micromass, Manchester, UK). The HRTOF-MS spectra were obtained on a Micromass LCT equipped with an electrospray ion (ESI) source. Optical rotations were measured with a JASCO DIP-370 spectropolarimeter (JASCO, Tokyo, Japan). IR spectra were obtained on a JASCO FT/IR-8900 spectrometer. The UV spectra were recorded on a Shimadzu UV-265FW spectrometer (Shimadzu, Kyoto, Japan). GC/MS analyses were carried out using an Agilent GC/MSD spectrometer (Agilent, Santa Clara, CA, USA; EI-MS detector, 5973; GC system, 6890; carrier gas, He). The LC/MS analyses were carried out using an Agilent LC/MSD equipped with an ESI ion source. The analytical HPLC operations were performed on an Agilent HP1100 HPLC system. The HPLC analyses were measured on an Agilent 1100 system (column, Cadenza CD-C18 (4.6×75 mm, Imtakt Co., Japan); mobile phase, A: 0.1% trifluoroacetic acid, B: acetonitrile, 65–95% B (0–15 min), 95% B (15–20 min), linear gradient, flow rate, 0.6 ml min^{-1}; detection UV 240 nm).

GC/MS analysis of components prepared from haplofungins by methanolysis

The compound **1** was treated with methanol containing 5% HCl at 80°C for 4 h in a sealed ampoule, and the reaction mixture was dried over a stream of N_2 gas. The residue was then dissolved in pyridine and BSTFA was added. The mixture was allowed to stand at room temperature for 5 min. The product was subsequently subjected to GC/MS analysis under the following conditions: HP-5 MS capillary column (Agilent, 0.25×30 mm). The column temperature was programmed to rise 5°C min^{-1} from 120°C to 240°C. The flow rate of the He carrier gas was 1.5 ml min^{-1}. The retention times of the TMS-derivatives are shown in Table 4. Authentic D-arabinono-1,4-lactone was prepared from D-arabinose by oxidation according to the method of Han *et al.*[10]

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