ORIGINAL ARTICLE

Haplofungins, novel inositol phosphorylceramide synthase inhibitors, from *Lauriomyces bellulus* SANK 26899 III. Absolute structure of haplofungin A

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The absolute structure of haplofungin A, a potent fungal inositol phosphoceramide (IPC) synthase inhibitor from *Lauriomyces bellulus* SANK 26899, was determined by chiral GC/MS analysis, NMR and X-ray crystallographic analysis of the derivatives of degradation products.

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INTRODUCTION

Haplofungins were isolated from the fungus strain *Lauriomyces bellulus* SANK 26899 and were found to show potent inhibitory activities against fungal inositol phosphoceramide (IPC) synthase. In the preceding papers, we described the taxonomy of the strain SANK 26899 and its production, isolation, biological activities¹ and physico-chemical properties as well as the planar structure of haplofungin A.² The structures of haplofungins are composed of an arabinonic acid moiety and an unsaturated β -ketoacyl moiety and some of these partial structures are in common with the reported IPC synthase inhibitors, rustmicin^{3,4} and khafrefungin.^{5,6} Moreover, it is supposed that the structures of haplofungins mimic that of phytoceramide, which is the substrate of IPC synthase. Therefore, information on the absolute structure of haplofungins would be very useful not only for the study of the structure–activity relationship but also for the design of new antifungal drugs based on haplofungins.

In this paper, we report the absolute structure of haplofungin A (**1**), the most potent IPC synthase inhibitor among the reported natural products.

RESULTS AND DISCUSSION

The strategy for determination of the absolute configuration of **1** is summarized in Figure 1.

As **1** could not be crystallized in all the solvents tested, stereochemical studies were performed on the two composition elements, an arabinonic acid moiety and a long-chain β -keto acid moiety. As the aldonic acid moiety in **1** was identified earlier as arabinonic acid,² its absolute configuration in the moiety was assumed as a result of chiral GC/MS analysis. In contrast, that of the long-chain β -keto acid was elucidated by NMR and an X-ray crystallographic analysis.

Stereochemistry of the arabinonic acid moiety

To determine the absolute configuration of the arabinonic acid moiety, the trifluoroacetyl (TFA) derivative (**1c**) prepared from the hydrolysate (**1a**) was analyzed by gas chromatography using a chiral column (HP-Chiral-20 β capillary column, Agilent Technologies, Santa Clara, CA, USA). As shown in Figure 2, the peak with a retention time of 21.55 min was identical to that of authentic trifluoroacetyl-D-arabinono-1,4-lactone prepared from D-arabinonic acid.⁷ Thus, the aldonic acid moiety in **1** was determined to be D-arabinonic acid.

Absolute configuration of the secondary alcohol at C-13

To determine the absolute configuration of position 13 in the alkyl chain of **1**, we chose α -methoxy-2-naphthylacetic acid (2-NMA) as an anisotropic reagent.⁸ 2-NMA is known as a very suitable reagent for the configurational analysis of secondary alcohol in a long alkyl chain. The lipid moiety **1b** was prepared from **1** by methanolysis through the decarboxylation at C-1. **1b** was treated with (R)- or (S)-2-NMA, triethylamine, 2,4,6-trichlorobenzoyl chloride and 4-(dimethylamino)pyridine (DMAP) in THF at room temperature for 1 h to give (R)- or (S)-2-NMA ester derivatives (**1d**, **1e**). ¹H NMR signals of **1d** and **1e** were assigned by 1D and 2D NMR experiments. As shown in Figure 3, completely opposite signs of $\Delta\delta$ values ($=\delta_R-\delta_S$), which were systematically oriented in the right and left sides of C-13, were observed. This anisotropic effect clearly indicated that the configuration of C-13 was S.

Absolute structure of the long alkyl chain moiety of **1**

Among the three undetermined chiral centers (C-2, C-6 and C-8), C-2 is an α -carbon of β -keto acid. It can easily be racemized under conventional hydrolysis conditions. Moreover, decarboxylation

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Figure 1 Planar structure of haplofungin A and the elucidation strategy for the stereochemistry.

Figure 2 Chiral GC/MS analysis of trifluoroacetylated hydrolysate. (a) Authentic D- and L-arabinono-1,4-lactone. (b) Hydrolysate prepared from **1**.

Figure 3 $\Delta\delta$ values obtained from **1d** and **1e**.

Figure 4 ORTEP diagram and structure of (*R*)-PGME-amide derivative (**1g**).

occurred at C-1 under acidic conditions, with the disappearance of chirality at C-2.

To avoid such decomposition, C-3 carbonyl was converted to a corresponding alcohol before alkaline hydrolysis. For this purpose, **1** was treated with CeCl_3 and NaBH_4 in methanol⁹ to selectively reduce C-3 carbonyl and the reaction was accomplished without the racemization at C-2. Following hydrolysis by 0.1 N NaOH gave **1f** as a single isomer. Subsequently, (*R*)-phenylglycine methylester (PGME) derivative (**1g**) was obtained by the condensation of **1f** and (*R*)-PGME in DMF. **1g** was crystallized by the hanging drop vapor diffusion method from 75% methanol solution and X-ray crystal structure analysis of **1g**, unequivocally confirming the substructure of **1** determined by the NMR experiments.² As the absolute configuration of the PGME moiety was *R*, the absolute chemistries of C-2, C-6, C-8 and C-13 of **1** were determined to be 2*R*, 6*R*, 8*S* and 13*S*, respectively (Figure 4), and the absolute chemistry of C-13 was identical with the result determined by NMR experiments with the 2-NMA derivatives mentioned earlier.

Together with the absolute stereochemistry of the arabinonic acid moiety as described above, the absolute structure of **1** was established

to be 2*R*, 6*R*, 8*S*, 13*S*, 2'*S*, 3'*S* and 4'*R*, as shown in Figure 5. Haplofungins are related to khafrefungin, which is an IPC synthase inhibitor isolated by the Merck Group in 1997.⁵ Khafrefungin is composed of one aldonate moiety and one hydroxylated fatty acid moiety and its absolute chemistry has been determined by total synthesis.⁶ Although the absolute configuration of the arabinonic acid in **1** is consistent with that of khafrefungin, the stereochemistry of each corresponding hydroxylated carbon in the fatty acid was different. In an SAR study of khafrefungin,¹⁰ it has been reported that the stereochemistry of the methyl group at C-4 in the alkyl chain and the aldonic acid moiety were indispensable for its biological activities. In haplofungins, the importance of the stereochemistry of the aldonic acid moiety is thought to be the same as that of khafrefungin. This information suggests that the absolute structure of the aldonic acid moiety of these molecules might mimic the hydrophilic terminal of the widely distributed (2*R*, 3*S*, 4*S*)-form phytosphingosine in the plant kingdom. The stereochemistry at C-2 was determined to be *R* by the selective reduction of C-3 keto carbonyl, the PGME derivatization and X-ray structural analysis of the derivative. An α -methyl- β -keto acid

Figure 5 The absolute configuration of haplofungin A (1), khafrefungin and rustmicin.

moiety is a common substructure of haplofungins and rustmicin, isolated from a cultured broth of *Micromonospora chalicea* 980-MC as IPC synthase inhibitor, and the stereochemistry of the unit is also the same^{3,4} If the long-chain moiety between C-1 and C-13 of haplofungins were macrolactonized, the carbon skeleton of the product would have the same 14-membered macrolide ring system as that of rustmicin. The configuration of C-2 in rustmicin is essential for its inhibitory activity against IPC synthase.⁴ If the substructure of haplofungin acts on the active site of IPC synthase on which rustmicin acts, the stereochemistry of C-2 in haplofungin would be very important for the binding affinity. Haplofungins are unique hybrid compounds that have the dual structural features of two known IPC synthase inhibitors. It is expected that such stereochemical information will contribute to SAR studies using organic synthesis based on the frame of haplofungins to create effective antifungal agents.

EXPERIMENTAL SECTION

Materials

Haplofungin A (1) was purified as described in a preceding paper. 2-Naphthaldehyde was purchased from Sigma-Aldrich (St Louis, MO, USA). Trifluoroacetic anhydride was purchased from Kanto Chemical, Tokyo, Japan. (*R*)-Phenylglycine methyl ester was purchased from Tokyo Chemical Industry, Tokyo, Japan.

General experimental procedures

1D and 2D NMR spectra were recorded on a Bruker AVANCE 500 spectrometer (Bruker, Rheinstetten, Germany) equipped with a Bruker cryoprobe. The data set acquired for each sample consisted of 1D ¹H, ¹³C NMR, gs-DQF-COSY, gs-HSQC, gs-HMBC and gs-HSQC-HOHAHA experiments using Bruker standard microprograms. The GC/MS analysis was performed using an Agilent GC/MSD spectrometer (EI-MS detector, 5973; GC system, 6890; carrier gas, He). The LC/MS analysis was performed using an Agilent LC/MSD equipped with an electrospray ion source. Analytical HPLC was performed on an Agilent HP1100 HPLC system.

Chiral GC/MS analysis of arabinonic acid moiety in 1

Compound 1 (0.5 mg) was dissolved in 5% methanolic hydrochloride (200 μ l), and methanolysis was performed in a sealed ampoule at 105 °C for 8 h. After the reaction mixture was dried under a stream of N₂ gas, the residue was dissolved in CH₂Cl₂ (100 μ l) and trifluoroacetic anhydride (100 μ l) was added. The mixture was allowed to stand at room temperature for 5 min. The product was subsequently applied to chiral GC/MS analysis under the following conditions: HP-Chiral-20 β capillary column (0.25 $\times$ 30 mm, Agilent Technologies) coated with 0.25 mm film thickness. The column temperature was programmed to rise in the ratio of 4 °C/min from 70 to 150 °C and then to rise to 190 °C. The flow rate of the He carrier gas was 1.5 ml min⁻¹. When the authentic pertrifluoroacetyl-D,L-arabinono-1,4-lactone showed the retention times of 21.54 and 21.93 min, the product 1c obtained from 1 was eluted at 21.55 min.

Preparation of 2-NMA esters from 1

α -Methoxy-2-naphthylacetic acid was obtained as follows: 2-naphthaldehyde was treated with bromoform and KOH in methanol to obtain the racemic 2-NMA, according to the method of Jiang and Compere.¹¹ After esterification with (–)-menthol, the diastereoisomeric esters were purified by preparative HPLC (column, Develosil C30 UG-5 (20 $\times$ 150 mm, Nomura Chemical, Aichi, Japan); mobile phase, 85% aqueous methanol; flow rate, 11.0 ml min⁻¹; detection, UV 310 nm). Each diastereomer was dissolved in 4 N HCl/dioxane and refluxed for 10 h. The mixture was purified by HPLC (column, Pegasil ODS (20 $\times$ 250 mm, Senshu Scientific, Tokyo, Japan); mobile phase, A, 0.1% phosphoric acid, B, 90% acetonitrile containing 0.1% phosphoric acid; linear gradient program from 30 to 100% of B in A in 30 min; flow rate, 10.0 ml min⁻¹; detection, UV 310 nm) to give each enantiomer, (*S*)- and (*R*)-2-NMA. Each optical rotation was as follows: (*S*)-2-NMA: [α]_D²⁴ +100° (c 1.3, methanol). Lit. [α]_D²⁵ +100.5° (c 0.63, methanol)⁸ (*R*)-2-NMA: [α]_D²⁴ -110° (c 1.0, methanol). Lit. [α]_D²⁵ -138.7° (c 0.35, methanol).⁸ 1 (50 mg, 0.08 mmol) was refluxed with 5% methanolic hydrochloride (10 ml) for 4 h and the mixture was evaporated *in vacuo* to dryness. The residue was extracted with ethyl acetate, and washed with brine. The organic layer was dried over anhydrous Na₂SO₄ and concentrated *in vacuo* to give a crude mixture containing 1b (29 mg, 87.5%). A dry THF solution (2 ml) containing the crude 1b (25 mg, 0.06 mmol), (*R*)-2-NMA (14.6 mg, 0.07 mmol), triethylamine (17 μ l, 0.12 mmol), DMAP (15 mg, 0.123 mmol) and 2,4,6-trichlorobenzoyl chloride

(11 μ l, 0.07 mmol) was stirred at room temperature for 1 h. After dilution with toluene (20 ml), the mixture was stirred again at room temperature for 1 h followed by filtration and concentration. The resulting residue was purified by preparative HPLC (column, Develosil C30UG-5, Nomura Chemical (20 $\times$ 150 mm); mobile phase, methanol; flow rate, 10.0 ml min⁻¹; detection, UV 254 nm) to afford the (*R*)-2-NMA ester (**1d**, 10.2 mg, 33%). (*S*)-2-NMA ester (**1e**, 8.3 mg, 16.7%) was prepared by the same method.

Preparation of (*R*)-PGME amide from **1**

The carbonyl group at C-3 in **1** was reduced by the method of Larock *et al.*⁹ **1** (140 mg, 0.23 mmol) was dissolved in methanol (5 ml) containing CeCl₃ (280 mg), and NaBH₄ (30 mg) was slowly added by stirring. The mixture was allowed to react at room temperature for 10 min followed by neutralization with 0.1 N HCl. The mixture was evaporated *in vacuo* to remove the methanol and the aqueous suspension was extracted with ethyl acetate (10 ml). The organic layer was dried over anhydrous Na₂SO₄ and evaporated *in vacuo*. Then, the crude mixture was dissolved in acetone (5 ml) and treated with 0.1 N NaOH (500 μ l) at room temperature for 5 min. The mixture was neutralized with 0.1 N HCl and extracted with ethyl acetate (50 ml). The organic layer was dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by preparative HPLC (column, Capcell Pak C8UG-120, Shiseido Co. Ltd., Tokyo, Japan (20 $\times$ 250 mm); mobile phase, 90% aqueous acetonitrile containing 0.1% phosphoric acid; flow rate, 12.0 ml min⁻¹; detection, UV 210 nm). The fraction was concentrated *in vacuo* and extracted with ethyl acetate. The organic layer was dried over anhydrous Na₂SO₄ and concentrated *in vacuo* to give a reduced hydrolysate (**1f**, 70.8 mg, 37%). To a stirred dry DMF solution (5 ml) of **1f** (70 mg, 0.15 mmol) and (*R*)-PGME (22.0 mg, 0.13 mmol) were successively added EDC hydrochloride (30 mg, 0.16 mmol) and DMAP (3 mg, 0.025 mmol) followed by standing at room temperature for 8 h. Then, the reaction mixture was extracted with ethyl acetate and the organic layer was successively washed with 5% HCl, saturated NaHCO₃ solution and brine. The organic layer was dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The resulting residue was purified by preparative HPLC (column, Develosil C30 UG-5 (20 i.d. $\times$ 150 mm); mobile phase, methanol—5 mM HCOONH₄ (93:7); flow rate, 12.0 ml min⁻¹; detection, UV 210 nm) to afford the (*R*)-PGME amide derivative (**1g**, 59.0 mg, 67%).

X-ray crystallographic analysis of **1g**

Compound **1g** was crystallized by the hanging drop vapor diffusion method from 75% methanol solution at room temperature. Crystal with the approximate dimensions of 0.5 $\times$ 0.3 $\times$ 0.04 mm was used for the X-ray analysis. All measurements were made on an AFC-7 diffractometer (Rigaku Corporation, Tokyo, Japan) at -150 °C with graphite monochromated Cu-K α radiation. The crystal data are as follows: C₃₇H₆₃NO₅, MW 601.91, orthorhombic, space

group *P*2₁2₁2₁. *a*=20.565(3) Å, *b*=34.364(8) Å, *c*=5.163(2) Å, *Z*=4, *D*_c=1.096 g cm⁻³. The structure was solved by a direct method with the program SIR88.¹² The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included as isotropical but not refined. The final cycle of full-matrix least squares refinement was based on 1234 observed reflections (*I*>3.0 σ (*I*)) and converged with *R*=7.0% and *R*_w=9.6%, where *w*=1/ σ ²(*F*_o).

Supplementary Information

The Crystal Data Form can be found in the supplementary information in the online version.

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