JOC The Journal of Organic Chemistry

Article

pubs.acs.org/joc

Simplifungin and Valsafungins, Antifungal Antibiotics of Fungal Origin

Hiroyuki Ishijima,[†] Ryuji Uchida,[†] Masaki Ohtawa,[†] Ariko Kondo,[†] Kenichiro Nagai,[†] Keisuke Shima,[§] Kenichi Nonaka,[‡] Rokuro Masuma,[‡] Susumu Iwamoto,[∥] Hideyuki Onodera,[∥] Tohru Nagamitsu,[†] and Hiroshi Tomoda*[,†]

[†]Graduate School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

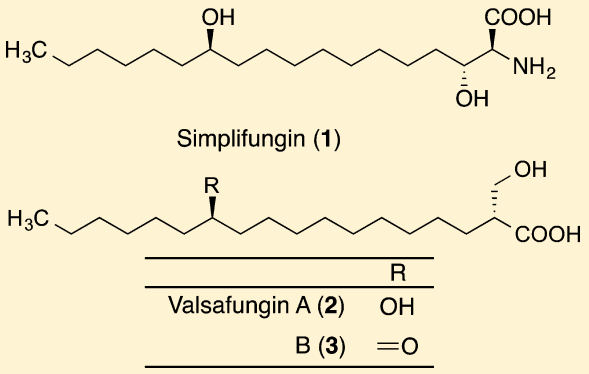
[‡]Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

[§]Life Science Business Department, Shimadzu Corporation, Kyoto 604-8511, Japan

[∥]Chemical Research Laboratories, R&D Division, Kyowa Hakko Kirin Co., Ltd., 1188 Shimotogari, Nagaizumi-cho, Shizuoka 411-8731, Japan

Supporting Information

ABSTRACT: The targets of antifungal antibiotics in clinical use are more limited than those of antibacterial antibiotics. Therefore, new antifungal antibiotics with different mechanisms of action are desired. In the course of our screening for antifungal antibiotics of microbial origins, new antifungal antibiotics, simplifungin (**1**) and valsafungins A (**2**) and B (**3**), were isolated from cultures of the fungal strains *Simplicillium minatense* FKI-4981 and *Valsaceae* sp. FKH-53, respectively. The structures of **1** to **3** including their absolute stereochemistries were elucidated using various spectral analyses including NMR and collision-induced dissociation (CID)-MS/MS as well as chemical approaches including modifications to the Mosher's method. They were structurally related to myriocin. They inhibited the growth of yeast-like and zygomycetous fungi with MICs ranging between 0.125 and 8.0 μg/mL. An examination of their mechanisms of action by the newly established assay using LC−MS revealed that **1** and **2** inhibited serine palmitoyltransferase activity, which is involved in sphingolipid biosynthesis, with IC_{50} values of 224 and 24 nM, respectively.



INTRODUCTION

Over the past 30 years, the number of invasive fungal infections has continued to persist, and this is primarily due to the increased use of immunosuppressive drugs, radiotherapy, parenteral nutrition, and antitumoral chemotherapy in highly advanced medical treatments. The fungal infections caused by *Candida*, *Aspergillus*, *Cryptococcus*, and *Rhizopus* species pose a particular threat to patients with opportunistic infections. The antifungal agents in current clinical use have mainly been classified into four types based on their modes of action:[1] azoles, which inhibit the enzyme lanosterol 14 α-demethylase in fungal ergosterol biosynthesis; polyenes, which physically interact with fungal sterols in cell membranes; candins, which inhibit the enzyme 1,3-β-D-glucan synthase; and 5-fluorocytosine, which inhibits fungal DNA synthesis. The number of antifungal agents is markedly lower than that of antimicrobial agents, and these drugs have serious side effects and have been implicated in the emergence of drug-resistant fungi.[2] The recent development of new antifungal drugs has mainly been limited to improving azole derivatives.[3] Challenges are associated with the discovery of new antifungal leads. However, new antifungal agents with different mechanisms of action are greatly desired.

On the basis of this background, we screened the secondary metabolites of microorganisms for new antifungal antibiotics. Natural products including microbial metabolites have diverse chemical characteristics and unique biological activities, thereby providing new leads and targets for the development of antifungal agents. In the course of our screening program, two fungal strains FKI-4981 and FKH-53, identified as *Simplicillium minatense* and *Valsaceae* sp., were found to produce new antifungal antibiotics designated as simplifungin (**1**) and valsafungins (**2** and **3**) (Figure 1), respectively. In the present study, the structural elucidation, biological properties, and modes of action of **1** to **3** are described.

RESULTS

Structural Elucidation of Simplifungin. Simplifungin (**1**) was isolated as a white powder, and its molecular formula was determined to be $C_{18}H_{37}NO_4$ (calcd for $C_{18}H_{38}NO_4$, 332.2801) based on a high-resolution ESI-MS $[M + H]^+$ of 332.2809, indicating one degree of unsaturation. The IR spectrum showed

Received: April 26, 2016
Published: July 11, 2016

DOI: 10.1021/acs.joc.6b00952
J. Org. Chem. 2016, 81, 7373−7383

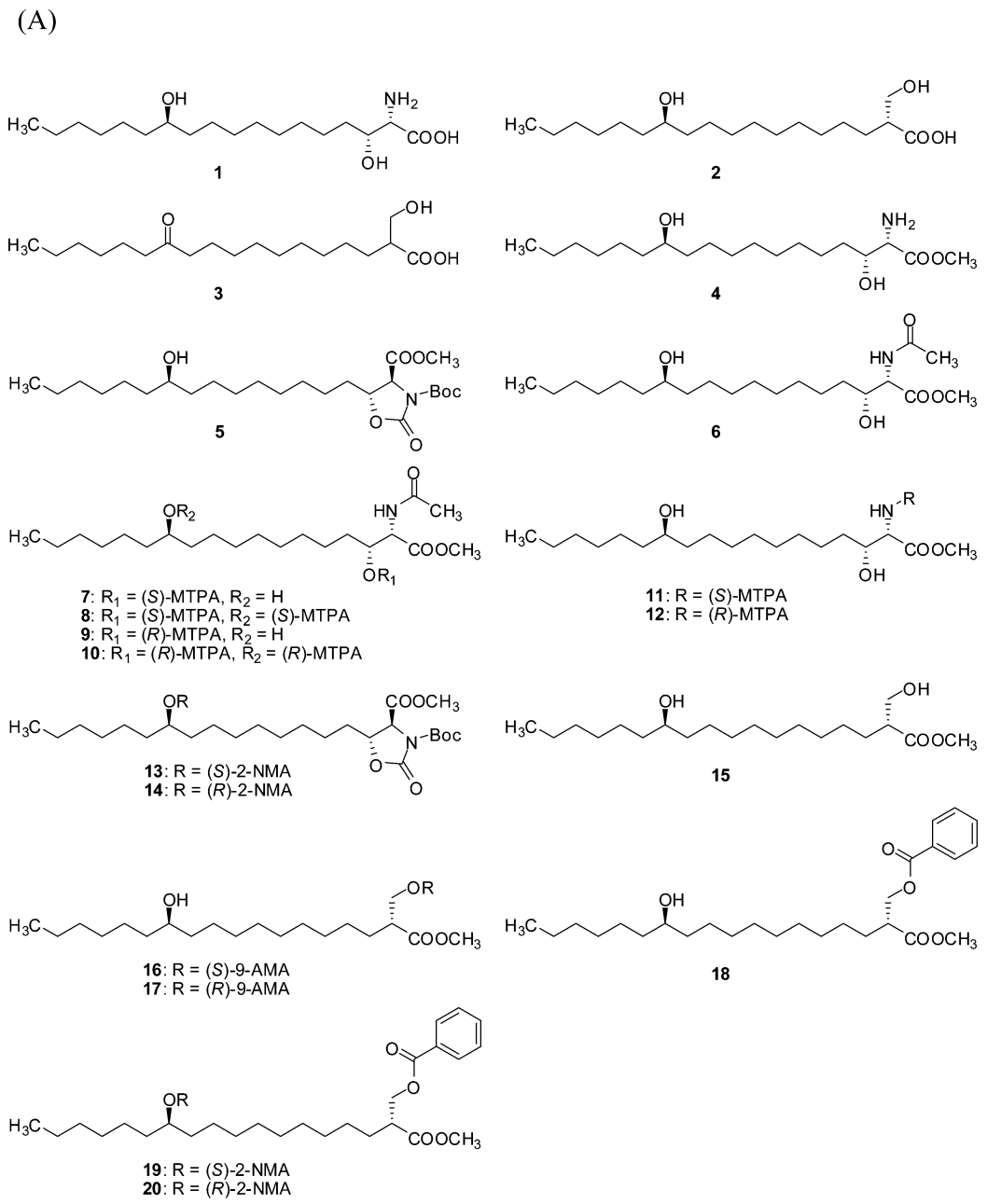


Figure 1. Structures of simplifungin (**1**), valsafungins A (**2**) and B (**3**), and their derivatives (**4**–**20**).

absorption maxima at 1677 and 3425 cm^{-1}, suggesting the presence of a carbonyl moiety and a hydroxyl and amino moiety, respectively. As shown in Table 1, the ^1H NMR spectrum (in methanol-d_4) showed 32 proton signals, which were classified into one methyl proton, 13 sp^3 methylene protons, and three oxygenated or nitrogenated sp^3 methine protons. The ^{13}C NMR spectrum (in methanol-d_4) showed 18 resolved signals, which were classified into one methyl carbon, 13 sp^3 methylene carbons, three oxygenated or nitrogenated sp^3 methine carbons, and one carbonyl carbon by an analysis of DEPT. The connectivity of proton and carbon atoms was established by the HMQC spectrum. An analysis of the $^1H-^1H$ COSY and $^{13}C-^1H$ long-range couplings of ^2J and ^3J observed in the HMBC spectrum gave the following three partial structures I to III, as shown in Figure 2A. (1) The $^1H-^1H$ COSY correlations between the methine proton H-2 (δ 3.87) and methine proton H-3 (δ 4.11), between H-3 and the methylene protons H_2-4 (δ 1.58) and between H_2-4 and the methylene protons H_2-5 (δ 1.37) in combination with the HMBC correlation from H-2 and H-3 to the carbonyl carbon C-1 (δ 170.7) revealed the partial structure I. In addition, ^1H and ^{13}C NMR chemical shifts at C-2 (δ_H 3.87, δ_C 58.6) and C-3 (δ_H 4.11, δ_C 70.1) suggested that the amino moiety and hydroxyl moiety were connected to the methine carbon C-2 and methine carbon C-3, respectively. (2) The $^1H-^1H$ COSY correlations between the methylene protons H_2-11 (δ 1.42) and the methylene protons H-12 (δ 3.50) and between H-12 and the methylene protons H_2-13 (δ 1.42) in combination with the HMBC correlations from H-12 to the sp^3 methylene carbon C-10 (δ 26.8) and the sp^3 methylene carbon C-14 (δ 26.8) and from H_2-13 to C-14 revealed the partial structure II. Furthermore, ^1H and ^{13}C chemical shifts at C-12 (δ_H 3.50, δ_C 72.5) suggested that the hydroxyl moiety was connected to the sp^3 methine carbon C-12. (3) The $^1H-^1H$ COSY correlation between the methylene protons H_2-17 (δ 1.32) and methyl protons H_3-18 (δ 0.91) in

DOI: 10.1021/acs.joc.6b00952
J. Org. Chem. 2016, 81, 7373−7383

Table 1. ^{1}H and ^{13}C NMR Chemical Shifts in 1 to 3

	1[a]		2[a]		3[b]	
position	δ_C, mult[c]	δ_H, (mult, J in Hz)[d]	δ_C, mult[c]	δ_H, (mult, J in Hz)[d]	δ_C, mult[c]	δ_H, (mult, J in Hz)[d]
1	170.7, C	–	178.6, C	–	179.1, C	–
2	58.6, CH	3.87 (d, 3.6)	49.8, CH	2.50 (ddt, 8.5, 8.0, 5.5)	50.1, CH	2.48 (m)
3	70.1, CH	4.11 (ddd, 7.2, 3.6, 3.6)	29.7, CH_2	1.53 (m)	29.8, CH_2	1.52 (m)
4	35.2, CH_2	1.58 (m)	28.4, CH_2	1.32 (m)	29.9, CH_2	1.29 (m)
5	26.7, CH_2	1.37 (m)	30.8, CH_2[h]	1.31 (m)[m]	28.4, CH_2	1.33 (m)
6	30.68, CH_2[e]	1.35 (m)[l]	30.55, CH_2[h]	1.31 (m)[m]	30.5, CH_2[j]	1.30 (m)[n]
7	30.4, CH_2[e]	1.35 (m)[l]	30.72, CH_2[h]	1.31 (m)[m]	30.3, CH_2[j]	1.30 (m)[n]
8	30.8, CH_2[e]	1.35 (m)[l]	30.55, CH_2[h]	1.31 (m)[m]	30.5, CH_2[j]	1.30 (m)[n]
9	30.60, CH_2[e]	1.35 (m)[l]	30.65, CH_2[h]	1.31 (m)[m]	30.7, CH_2[j]	1.30 (m)[n]
10	26.80, CH_2[f]	1.44 (m)	26.8, CH_2	1.43 (m)	24.90, CH_2[k]	1.54 (m)
11	38.5, CH_2[g]	1.42 (m)	38.44, CH_2[i]	1.37 (m); 1.42 (m)	48.5, CH_2	2.44 (t, 7.3)
12	72.5, CH	3.50 (m)	72.5, CH	3.50 (m)	214.4, C	–
13	38.4, CH_2[g]	1.42 (m)	38.41, CH_2[i]	1.37 (m); 1.42 (m)	48.5, CH_2	2.44 (t, 7.3)
14	26.78, CH_2[f]	1.32 (m)	26.8, CH_2	1.31 (m)	24.88, CH_2[k]	1.54 (m)
15	30.56, CH_2[e]	1.35 (m)[l]	30.63, CH_2[h]	1.31 (m)[m]	30.5, CH_2[j]	1.30 (m)[n]
16	33.1, CH_2	1.31 (m)	33.1, CH_2	1.30 (m)	32.8, CH_2	1.29 (m)
17	23.7, CH_2	1.32 (m)	23.7, CH_2	1.30 (m)	23.6, CH_2	1.32 (m)
18	14.4, CH_3	0.91 (t, 7.3)	14.4, CH_3	0.90 (t, 7.0)	14.3, CH_3	0.90 (t, 7.0)
1′	–	–	64.2, CH_2	3.62 (dd, 11.0, 5.5); 3.70 (dd, 11.0, 8.0)	64.3, CH_2	3.60 (dd, 10.5, 5.5); 3.70 (dd, 10.5, 8.0)

[a]Spectra were recorded at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR using MeOH-d_4 as a solvent. [b]Spectra were recorded at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR using MeOH-d_4 as a solvent. [c]Chemical shifts are shown with reference to methanol-d_4 as 49.0 ppm. [d]Chemical shifts are shown with reference to methanol-d_4 as 3.31 ppm [e−k]Chemical shifts are exchangeable. [l−n]Overlapped signals.

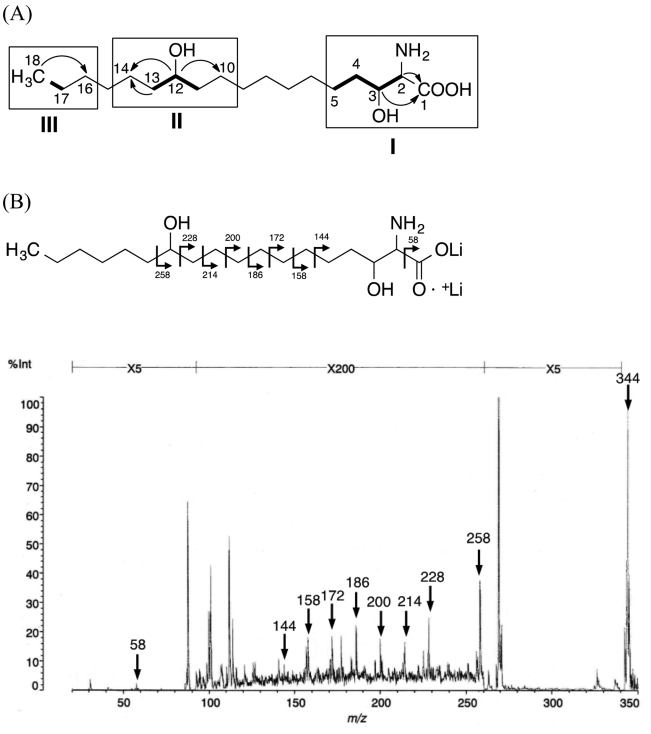


Figure 2. Structural elucidation of **1**. (A) Key correlations in ^{1}H−^{1}H COSY and HMBC experiments. (B) Product ion spectra of **1** by the CID-MS/MS experiment.

combination with the HMBC correlation from H_3-18 to the sp^3 methylene carbon C-16 (δ 33.1) revealed the partial structure III. Taking into consideration the molecular formula, the remaining structure consisted of five methylenes. However, it was not possible to define the position of a hydroxyl methine in the partial structure II from these data because the methylene proton signals overlapped. Therefore, an MS/MS experiment with a MALDI-TOF-TOF instrument was performed in order to produce high-energy collision-induced dissociation (CID) fragmentation ions that make it possible to determine the position of the C-12 hydroxyl methine. As shown in Figure 2B, the lithium ion adduct peak $[M - H + 2Li]^+$ was observed at m/z 344, and m/z increased regularly by 14 masses corresponding to CH_2 from m/z 144 to 228, while 30 masses corresponding to CH−OH increased from m/z 228 (C-11) to m/z 258 (C-13), revealing the position of the hydroxyl methine at C-12. Furthermore, the MS/MS spectrum fully supported the structure elucidated from the NMR data. Taken together, the planar structure of **1** was elucidated as shown in Figure 1, which fulfilled the molecular formula and degrees of unsaturation.

Stereochemistry of Simplifungin. In order to elucidate the relative stereochemistries of C-2 and C-3 in **1**, **1** was derivatized to the methyl ester **4**, followed by a reaction with $(Boc)_2O$[4] to give the oxazolidinone derivative **5**. The coupling constant between the annular protons of H-2 and H-3 in **5** was 4.5 Hz. Since the respective coupling constants between the two annular protons of *cis* and *trans* isomers are generally 6−9 Hz and 4−6 Hz, respectively,[5] the annular protons were likely to have been oriented to the *trans* position. NOE was not observed between H-2 and H-3, indicating that the relative stereochemistry is 2*S**, 3*R**.

The absolute stereochemistry of C-2 and C-3 was then elucidated by the modified Mosher's method.[6] Compound **4** was treated with acetyl chloride to yield **6**, which was modified with (*S*)- and (*R*)-methoxytrifluoromethylphenylacetic acid (MTPA) to yield four MTPA esters: **7** to **10**.[7] Of these, the mono MTPA derivatives **7** and **9** were purified by HPLC. The ^{1}H NMR spectra of **7** and **9** showed that the $\Delta\delta$ values ($\Delta\delta = \Delta\delta_S - \delta_R$) of H-3 and H_2-4 were positive, whereas those of H-2, NH, H-1′, and H-2″ were negative (Figure 3A), indicating that the absolute stereochemistry is 2*S*, 3*R*. In order to confirm the absolute stereochemistry, the (*S*)- and (*R*)-MTPA amides **11** and **12** were prepared from **4** according to Kusumi's method.[8] Under the

DOI: 10.1021/acs.joc.6b00952
J. Org. Chem. 2016, 81, 7373−7383

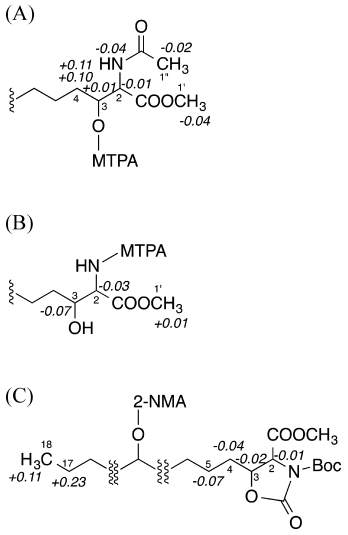


Figure 3. Absolute stereochemistry determination at C-2, C-3, and C-12 of **1**. (A) $\Delta\delta$ values ($\Delta\delta$ (in p.p.m.) = $\Delta\delta_S - \delta_R$) obtained for the MTPA esters **7** and **9**. (B) $\Delta\delta$ values ($\Delta\delta$ (in p.p.m.) = $\Delta\delta_S - \delta_R$) obtained for the MTPA amides **11** and **12**. (C) $\Delta\delta$ values ($\Delta\delta$ (in p.p.m.) = $\Delta\delta_R - \delta_S$) obtained for the 2-NMA esters **13** and **14**.

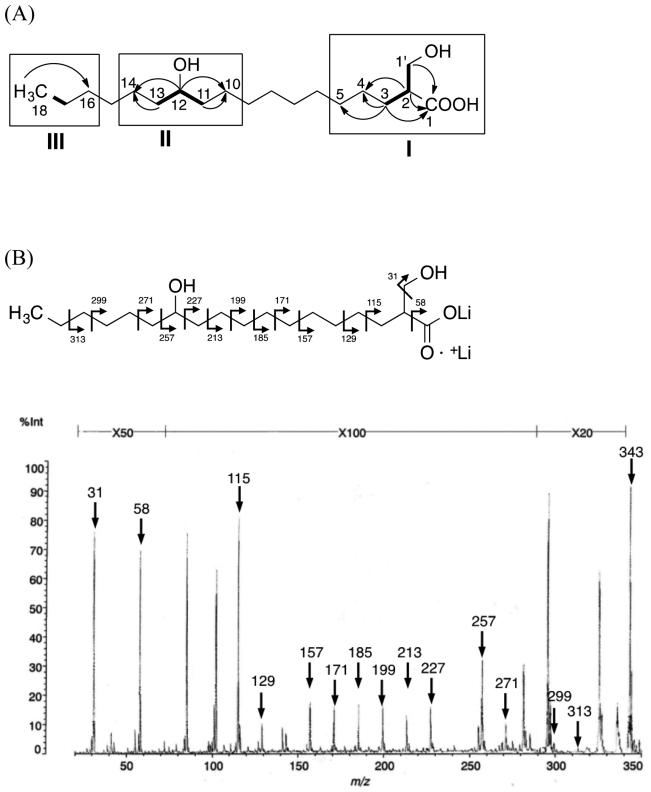


Figure 4. Structural elucidation of **2**. (A) Key correlations in ^{1}H−^{1}H COSY and HMBC experiments. (B) Product ion spreads of **2** by the CID-MS/MS experiment.

conditions employed, MTPA was selectively reacted with an amino residue to yield **11** and **12**. The ^{1}H NMR spectra of **11** and **12** indicated that the $\Delta\delta$ value ($\Delta\delta = \Delta\delta_S - \delta_R$) of H-1′ was positive, whereas those of H-2 and H-3 were negative (Figure 3B), supporting the above absolute stereochemistry. The absolute stereochemistry of C-12 was elucidated by preparing the (*S*)- and (*R*)-2-naphthylmethoxyacetic acid (2-NMA) esters **13** and **14** from **5** according to Kusumi's method.[9] The ^{1}H NMR spectra showed that the $\Delta\delta$ values ($\Delta\delta = \Delta\delta_R - \delta_S$) of H_2-17 and H_3-18 were positive, whereas those of H-2, H-3, H_2-4, and H_2-5 were negative (Figure 3C), indicating that the absolute stereochemistry of C-12 is 12*R*. Taken together, the complete structure of **1** including the absolute stereochemistry is shown in Figure 1.

Structural Elucidation of Valsafungins. Valsafungin A (**2**) was isolated as a white powder, and its molecular formula was determined to be $C_{19}H_{38}O_4$ (calcd for $C_{19}H_{39}O_4$, 331.2848) based on a high-resolution FAB-MS $[M + H]^+$ of 331.2845, indicating one degree of unsaturation. The IR spectrum showed absorption maxima at 1709 and 3400 cm^{-1}, suggesting the presence of a carbonyl moiety and hydroxyl moiety, respectively. As shown in Table 1, the ^{1}H NMR spectrum (in methanol-d_4) showed 35 protons signals, which were classified into one methyl proton, 14 sp^3 methylene protons, one sp^3 methine proton, one oxygenated sp^3 methylene proton, and one oxygenated sp^3 methine proton. The ^{13}C NMR spectrum (in methanol-d_4) showed 19 resolved signals, which were classified into one methyl carbon, 14 sp^3 methylene carbons, one oxygenated sp^3 methylene carbon, one sp^3 methine carbon, one oxygenated sp^3 methine carbon, and one carbonyl carbon by an analysis of DEPT. The connectivity of proton and carbon atoms was established by the HMQC spectrum. An analysis of ^{1}H−^{1}H COSY and the HMBC spectrum gave the following three partial structures I to III, as shown in Figure 4A. (1) The ^{1}H−^{1}H COSY correlation between the methine proton H-2 (δ 2.50) and the methylene protons H_2-3 (δ 1.53) and between H-2 and the oxygenated methylene protons H_2-1′ (δ 3.62, 3.70) in combination with the HMBC correlations from H_2-1′, H-2, and H_2-3 to the carbonyl carbon C-1 (δ 178.6), from H_2-2 and H_2-3 to the methylene carbon C-4 (δ 28.4), and from H_2-3 to the methylene carbon C-5 (δ 30.8) revealed the partial structure I. Furthermore, ^{1}H and ^{13}C chemical shifts at C-1′ (δ_H 3.62, 3.70, δ_C 64.2) suggested that the hydroxyl moiety was connected to C-1′. (2) The ^{1}H−^{1}H COSY correlations between the methylene protons H_2-11 (δ 1.37, 1.42) and the oxygenated methine proton H-12 (δ 3.50) and between H-12 and the methylene protons H_2-13 (δ 1.42), in combination with the HMBC correlations from H_2-11 and H-12 to the methylene carbon C-10 (δ 26.8) and from H-12 and H_2-13 to the methylene carbon C-14 (δ 26.8) revealed the partial structure II. Furthermore, ^{1}H and ^{13}C chemical shifts at C-12 (δ_H 3.50, δ_C 72.5) suggested that the hydroxyl moiety was connected to C-12. (3) The ^{1}H−^{1}H COSY correlation between the methylene protons H_2-17 (δ 1.32) and methyl protons H_3-18 (δ 0.91) in combination with the HMBC correlation from the methyl protons H_3-18 (δ 0.90) to the methylene carbon C-16 (δ 33.1) revealed the partial structure III. Taking into consideration the molecular formula, the remaining structure consisted of five methylenes. The position of a hydroxyl methine in the partial structure II was also assigned by MS/MS experiments with a MALDI-TOF-TOF instrument. As shown in Figure 4B, the lithium ion adduct peak $[M - H + 2Li]^+$ was observed at m/z 343, and CID-MS/MS fragmentations were observed at m/z 227 (C-11) and m/z 257 (C-13), revealing the position of the hydroxyl methine at C-12. Furthermore, the MS/MS spectrum fully supported the structure elucidated from NMR data. Taken together, the planar structure of **2** was elucidated as shown in Figure 1, which fulfilled the molecular formula and the degrees of unsaturation.

DOI: 10.1021/acs.joc.6b00952
J. Org. Chem. 2016, 81, 7373−7383

Valsafungin B (**3**) was isolated as a white powder. The structure of **3** was elucidated by comparing all spectroscopic data with those of **2**. The molecular formula was determined to be $C_{19}H_{36}O_4$ (calcd for $C_{19}H_{37}O_4$, 329.2692) based on a high-resolution FAB-MS $[M + H]^+$ of 329.2688, indicating that **3** was 2 mass units (H_2) smaller than **2**. The similarity in the NMR spectra to **2**, except for the signals of the oxygenated methine proton H-12 and oxygenated methine carbon at C-12 in **2** being missing and the ketone carbon C-12 (δ 214.4) appearing in **3** instead, suggested that **3** possessed a ketone residue instead of a hydroxyl methine residue at C-12. This assignment was supported by HMBC correlations from the methylene protons H_2-10 (δ 1.54), H_2-11 (δ 2.44), H_2-13 (δ 2.44), and H_2-14 (δ 1.54) to C-12.

Stereochemistry of Valsafungin A. Valsafungin A has two stereogenic centers at C-2 and C-12. Its absolute stereochemistry was investigated according to the modified Mosher's method. The valsafungin A methyl ester (**15**) was modified with (*S*)- and (*R*)-9-anthrylmethoxyacetic acid (9-AMA) to yield the respective 9-AMA esters **16** and **17** according to Ferreiro's method.[10] In this method, the hydroxyl moieties at C-1′ and C-12 were both potentially modified with 9-AMA; however, only the hydroxyl moiety at C-1′ was modified. The ^{1}H NMR spectra showed that the $\Delta\delta$ values ($\Delta\delta = \Delta\delta_R - \delta_S$) of H-2 and H_2-1″ were positive, whereas that of H_2-3 was negative (Figure 5A), indicating that

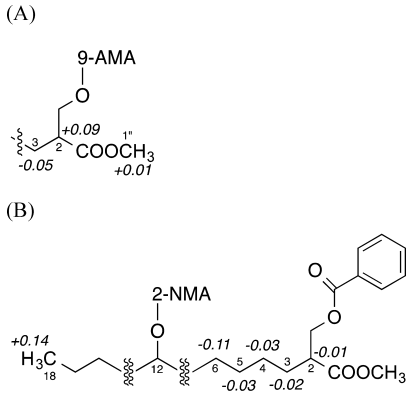


Figure 5. Absolute stereochemistry determination at C-2 and C-12 of **2**. (A) $\Delta\delta$ values ($\Delta\delta$ (in p.p.m.) = $\Delta\delta_R - \delta_S$) obtained for the 9-AMA esters **16** and **17**. (B) $\Delta\delta$ values ($\Delta\delta$ (in p.p.m.) = $\Delta\delta_R - \delta_S$) obtained for the 2-NMA esters **19** and **20**.

the absolute stereochemistry was elucidated to be 2*R*. Regarding the absolute stereochemistry at C-12, **15** was modified with benzoyl chloride to yield the benzoyl derivative **18**. **18** was then modified with (*S*)- and (*R*)-2-NMA to yield **19** and **20**, respectively, according to Kusumi's method.[9] The ^{1}H NMR spectra showed that the $\Delta\delta$ value ($\Delta\delta = \Delta\delta_R - \delta_S$) of H-18 was positive, whereas those of H-2, H_2-3, H_2-4, H_2-5, and H_2-6 were negative (Figure 5B), indicating that the absolute stereochemistry is 12*R*. Taken together, the complete structure of **2** including the absolute stereochemistry is shown in Figure 1. The C-12 absolute configuration of molecule **3** is assumed based on the stereochemistry of molecule **2**.

Antifungal Activities of Simplifungin, Valsafungins, and Their Derivatives. The MIC values of **1** to **6**, **15**, **18**, and myriocin against yeast-like, filamentous, and zygomycetous fungi are summarized in Table 2. Compound **1** showed antifungal activity (MIC, 4.0−8.0 μg/mL) against yeasts, except for *C. glabrata*, but no activity against filamentous and zygomycetous fungi. Compounds **2** and **3** strongly inhibited the growth of *C. albicans* (MIC, 0.5 μg/mL) and *C. parapsilosis* (MIC, 0.125 μg/mL). **3** was the most active against *Rhizopus* species (MIC, 0.5−1.0 μg/mL). Myriocin was the most active against yeasts (MIC, 0.125−0.5 μg/mL). Among the derivatives, only **4** exhibited antifungal activity against *C. albicans* (MIC, 0.25 μg/mL) and zygomycetous fungi (MIC, 4.0−32 μg/mL).

None of these compounds exhibited antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli*, or *Pseudomonas aeruginosa* up to 10 μg/6 mm paper disk.

Reversal of Antifungal Activity with Ketodihydrosphingosine and Dihydrosphingosine. Since the structures of **1** and **2** are similar to that of myriocin (see Figure 3S for the structure), enzymes involved in the sphingolipid biosynthetic pathway were speculated to be potential targets of **1** and **2**. The effects of **1** and **2** on the growth of *C. albicans* in the presence of dihydrosphingosine (DHS) and ketodihydrosphingosine (KDS), intermediates of the sphingolipid biosynthetic pathway, were investigated using two reported antifungal activity assays, methods A and B.[11] Preparation of KDS (**24**) is described in the Experimental Section and the synthetic sequence of reaction is depicted in Figure S4. In Method A, the addition of DHS as well as KDS was found to rescue *C. albicans* from growth inhibition by **1** and **2**. In Method B, the original IC_{50} value of **1** against *C. albicans* was 14.3 μM, which increased to 47.6 μM in the presence of KDS (14.0 μM) (Figure 6A). Compound **2** also showed similar anti-*C. albicans* activity in response to KDS. The IC_{50} values KDS-dependently increased from 0.74 μM (control) to 2.0 μM (with KDS 14 μM), 3.3 μM (28 μM), and 6.3 μM (56 μM) (Figure 6B). These results strongly suggested that **1** and **2** inhibit serine palmitoyltransferase (SPT) as well as myriocin (Figure S5).[12]

Inhibition of SPT with Simplifungin and Valsafungins. In order to determine whether **1**, **2** and **4** inhibit SPT, we established a new assay system. The SPT assay conditions were set according to Lester's method,[13] in which L-serine and palmitoyl-CoA were used as substrates and microsomes prepared from *S. cerevisiae* were used as an enzyme source. SPT activity in microsomes prepared from *C. albicans* appeared to be less potent than that from *S. cerevisiae*. Therefore, microsomes from *S. cerevisiae* were used for the new assay. After a 20 min incubation at 30 °C, the enzymatic product KDS was extracted and quantified by LC−MS, which detected at least 3.3 pmol. As shown in Figure 7A, the production of KDS decreased dose-dependently in the presence of **1** (0.03−1.0 μM), indicating that **1** inhibited SPT activity with an IC_{50} of 224 nM (Figure 7B). Under the same conditions, **2**, **4**, and myriocin inhibited SPT activity (*S. cerevisiae*) with IC_{50} values of 24.2, 45.4, and 11.8 nM, respectively (Figure 7B). The IC_{50} value of myriocin for SPT was similar to the value (IC_{50}; approximately 30 nM) reported by Sun et al.[14]

DISCUSSION

In the present study, the complete structures of fungal simplifungin (**1**) and valsafungin A (**2**) including their absolute stereochemistries were elucidated. Regarding **1**, the absolute stereochemistries of C-2 and C-3 were elucidated by synthesizing MTPA derivatives. Kusumi and co-workers reported that not only MTPA esters, but also MTPA amides can be used for elucidating absolute stereochemistries.[6,8] Accordingly, **6** was converted to the MTPA esters **7** and **9**, and **4** to the MTPA amides **11** and **12**. Under the conditions used, the mono-MTPA

DOI: 10.1021/acs.joc.6b00952
J. Org. Chem. 2016, 81, 7373−7383

Table 2. Antifungal Activities of Simplifungin (1), Valsafungins A (2) and B (3), Their Derivatives (4 to 6, 15, and 18), and Myriocin

	MIC (μg/mL)								
test organism	**1**	**2**	**3**	**4**	**5**	**6**	**15**	**18**	myriocin
Yeast-like fungus									
Candida albicans ATCC90029	8.0	0.50	0.50	0.25	>32	>32	>32	>32	0.25
Candida glabrata ATCC90030	>32	>32	>32	4.0	>32	>32	>32	>32	0.50
Candida parapsilosis ATCC90018	4.0	0.125	0.125	4.0	>32	>32	>32	>32	0.125
Cryptococcus neoformans ATCC90113	4.0	>32	>32	1.0	>32	>32	>32	>32	0.25
Saccharomyces cerevisiae S288C	8.0	16	>32	0.125	8.0	16	>32	>32	0.25
Filamentous fungus									
Aspergillus fumigatus NBRC33022	>32	>32	>32	>32	>32	>32	>32	>32	>16
Aspergillus flavas NBRC6243	>32	>32	>32	32	>32	>32	>32	>32	>16
Aspergillus niger NBRC105649	>32	>32	>32	>32	>32	>32	>32	>32	>16
Aspergillus terreus NBRC7078	>32	>32	>32	>32	>32	>32	>32	>32	>16
Zygomycetous fungus									
Rhizopus oryzae NBRC4705	32	2.0	0.50	32	>32	>32	>32	>32	8.0
Rhizopus microsporus IFM46417	>32	8.0	0.50	4.0	>32	>32	>32	>32	16
Rhizomucor pusillus NBRC9744	32	8.0	1.0	4.0	>32	>32	>32	>32	0.50
Absidia corymbifera IFM5335	>32	>32	>32	16	>32	>32	>32	>32	2.0

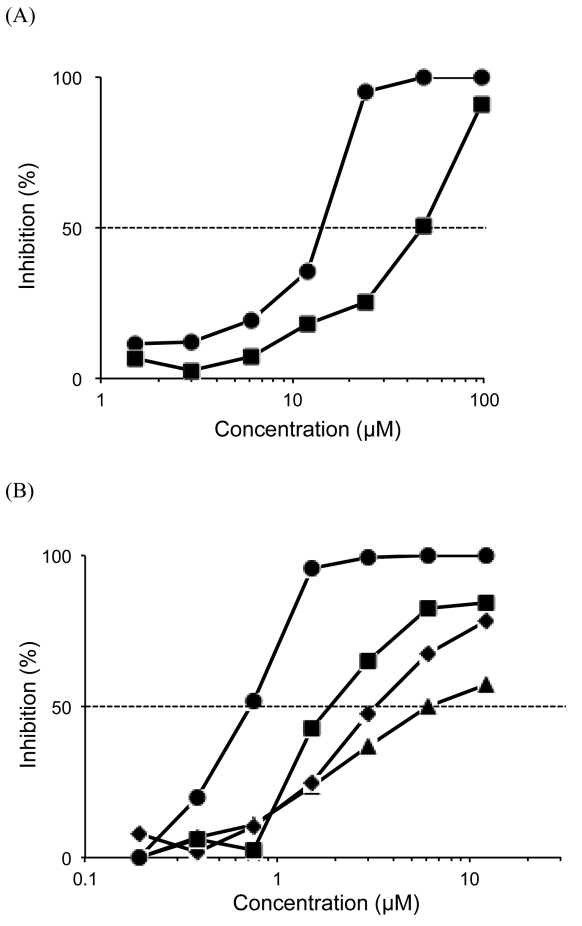


Figure 6. Reversal of anti-*C. albicans* activities of **1** (A) and **2** (B) by the addition of KDS. (A) ●, control (**1** alone); ■, + KDS (14.0 μM) (B) ●, control (**2** alone); ■, + KDS (14.0 μM); ♦, + KDS (28.0 μM); ▲, + KDS (56.0 μM).

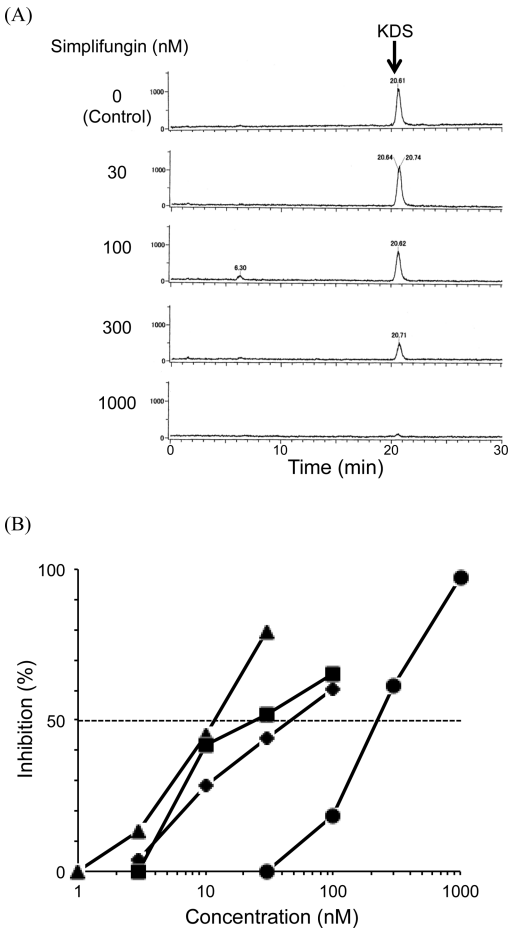


Figure 7. Inhibition of SPT activity. (A) LC−MS analysis of KDS. After microsomes (100 μg protein) prepared from *S. cerevisiae* were incubated with serine (25 mM) and palmitoyl-CoA (200 μM) at 30 °C for 20 min in the presence of **1** (0−1000 nM), the product (KDS) was analyzed by LC−MS (detailed conditions are described in the Experimental Section) (B) Inhibition of SPT activity by **1**, **2**, **4**, and myriocin for the SPT assay. ●, compound **1**; ■, compound **2**; ♦, compound **4**; ▲, myriocin.

derivatives **7** and **9** and di-MTPA derivatives **8** and **10** were obtained, indicating that the hydroxyl group at C-3 of **1** was more easily modified with the MTPA reagent than that at C-12. Analyses of the $\Delta\delta$ values from (mono-)MTPA esters and

DOI: 10.1021/acs.joc.6b00952
J. Org. Chem. 2016, 81, 7373−7383

MTPA amides (Figure 3A and 3B) resulted in the same conclusion that the absolute stereochemistry is 2*S*, 3*R*. The absolute stereochemistry of C-12 in **1** was elucidated by preparing the 2-NMA esters **13** and **14** from the oxazolidinone derivative **5**. Since the proton of C-12 is surrounded by a large number of methylene protons, 2-NMA derivatives are expected to be more suitable for analyzing $\Delta\delta$ values than MTPA derivatives. Anisotropic effects were observed in the protons H-2 to H-5 (Figure 3C), which are seven to 10 carbons away from this position. As a result, the absolute stereochemistry was elucidated to be 12*R*.

Regarding **2**, the absolute stereochemistry at C-2 of **2** was elucidated using 9-AMA reagents according to the method of Freire et al.[15] Methylated **2** (**15**) was converted to the 9-AMA esters **16** and **17**. An analysis of the $\Delta\delta$ values from 9-AMA esters indicated that the absolute stereochemistry is 2*R*. Furthermore, we confirmed the absolute stereochemistry at C-2 on the basis of the proposal of Freire et al.[10b] in order to assign the absolute stereochemistries of β-chiral primary alcohols. Namely, if the $\Delta\delta$ values of the nonpolar β-substituent protons are positive and the $\Delta\delta$ values of the proton attached to the asymmetric carbon (Cβ-H) and polar β-substituent protons are negative, the absolute stereochemistry of the asymmetric carbon is assigned an *S* configuration. On the other hand, if the $\Delta\delta$ values of Cβ-H and the polar β-substituent protons are positive and the $\Delta\delta$ values of the nonpolar β-substituent protons are negative, the absolute stereochemistry is assigned an *R* configuration. As for **2** (Figure 5A), the $\Delta\delta$ values of Cβ-H (H-2) and the polar β-substituent protons (H_3-1″) were positive and the $\Delta\delta$ value of the nonpolar β-substituent protons (H_2-3) was negative, indicating that the absolute stereochemistry at C-2 is *R*. The same conclusion was reached as that with Ferreiro's method,[10a] supporting the applicability of this method for elucidating the absolute stereochemistries of β-chiral primary alcohols. The absolute stereochemistry of C-12 in **2** was also elucidated by the same method as that used for **1** in order to prepare the 2-NMA esters **19** and **20** from **18**. Anisotropic effects were observed in H-2 to H-6 protons, which are six to 10 carbons away from this position. As a result, the absolute stereochemistry was elucidated to be 12*R*.

In the present study, the target enzyme underlying antifungal activities by simplifungin and valsafungins was determined. Based on their structural similarities to myriocin, the upper enzymes in sphingolipid biosynthesis were presumed to be a potential target of **1** and **2**. Namely, KDS is produced by SPT from L-serine and palmitoyl-CoA, and KDS is then reduced by 3-ketodihydrosphingosine reductase to yield DHS. Accordingly, the rescue assay was first performed using DHS and KDS. The anti-*C. albicans* activities of **1** and **2** were rescued by the addition of DHS and KDS to the medium (Figure 6), suggesting that the target enzyme was SPT. Therefore, the SPT assay was established according to the method reported by Miyake et al.[12a] with some modifications. [^{14}C]palmitoyl-CoA was initially used as a radiolabeled substrate instead of [^{14}C]serine. However, the amount of the reaction product [^{14}C]KDS was very low because [^{14}C]palmitoyl-CoA may be used in other metabolic pathways. We then attempted to quantify KDS by derivatizing it to dabsylated KDS using the method of Lester et al.[13] Dabsylated KDS was analyzed by LC/UV; however, it was difficult to quantify the amount. This may have been due to SPT activity in microsomes prepared from *C. albicans* being very low. A new assay method for SPT was established using *S. cerevisiae* microsomes as an enzyme source and quantifying the product KDS by LC−MS (Figure 7). In this method, a few picomoles (3.3 pmols) of KDS were detected. Several groups previously reported that myriocin inhibited SPT with an IC_{50} of approximately 30 nM in microsomes from *S. cerevisiae* using radiolabeled methods with [^{14}C]serine.[14] The IC_{50} of myriocin (15.4 nM) in our new assay was consistent with the above data.

In summary, valsafungins A (**2**) and B (**3**) and simplifungin methyl ester (**4**) showed strong antifungal activity against *C. albicans*, suggesting that these compounds can be potential leads for new antifungal agents. Furthermore, our new method for SPT assay will serve as a general and convenient analytical procedure for assessing the enzyme activity.

EXPERIMENTAL SECTION

General Procedures. Various NMR spectra were recorded on 400 or 600 MHz spectrometers in methanol-d_4 or chroloform-*d*. Chemical shifts are expressed in δ (ppm) and referenced to the residual solvent signals. High-resolution fast-atom bombardment (HR-FAB) and high-resolution electrospray ionization (HR-ESI) mass spectrometry data were recorded with high-resolution mass spectrometer. MS/MS experiment was performed with a MALDI-TOF-TOF instrument. Optical rotations were measured with a digital polarimeter. UV spectra were recorded on a spectrophotometer using MeOH as the solvent. IR spectra were recorded on a Fourier transform infrared spectrometer using KBr plates.

Fermentation and Isolation. The fungus FKI-4981 classified as *Simplicillium minatense*[16] was inoculated in a 500 mL Erlenmeyer flask containing 100 mL seed medium (2.0% glucose, 0.2% yeast extract, 0.05% $MgSO_4{\cdot}7H_2O$, 0.5% polypeptone, 0.1% KH_2PO_4, and 0.1% agar; pH 6.0). The flask was shaken on a rotary shaker at 27 °C for 3 days. The seed culture (1.0 mL) was transferred into a 500 mL Erlenmeyer flask ×48 containing 100 mL production medium (2.0% saccharose, 1.0% glucose, 0.5% corn steep powder, 0.5% meat extract, 0.1% KH_2PO_4, 0.3% $CaCO_3$, and 0.1% agar, pH 6.0). Fermentation was carried out under shaking conditions at 27 °C. After 3 days of fermentation, the culture broth (4.8 L) was centrifuged at 8000 rpm for 10 min to collect the mycelia. The cell body was treated with acetone (2 L). After the acetone extracts were filtered and concentrated, the resulting pellet was treated with $CHCl_3$. The $CHCl_3$ insoluble layer was collected and dried under reduced pressure to give brown materials (856 mg). Some of the materials (60 mg dissolved in 2.4 mL methanol) were finally purified by preparative HPLC under the following conditions: column, PEGASIL ODS SP100, 20 × 250 mm (Senshu Scientific Co., Tokyo, Japan); mobile phase, a 30 min gradient from 30% CH_3CN-0.05% TFA to 70% CH_3CN-0.05% TFA; detection, ELSD (Alltech, Nicholasville, Kentucky, United States) with a gas flow of 1.5 mL/min and tube temperature at 40 °C); flow rate, 8 mL/min. Under these conditions, **1** was eluted as a peak with a retention time of 17 min. The fraction was concentrated under reduced pressure to give pure **1** (18.1 mg) as a white powder.

*Simplifungin (**1**).* $[\alpha]^{23}_{D}$ + 1.4 (*c* 0.1, MeOH-0.05% TFA); IR (KBr) ν_{max} 3425, 2925, 2853, 1677, 1631, 1468, 1402, 1206, 1132 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESI-MS *m*/*z* 332.2788 [M + H]$^+$ (calcd for $C_{18}H_{38}NO_4$ *m*/*z* 332.2801).

The fungus FKH-53 was inoculated in a 500 mL Erlenmeyer flask containing 100 mL seed medium (2.0% glucose, 0.2% yeast extract, 0.05% $MgSO_4{\cdot}7H_2O$, 0.5% polypeptone, 0.1% KH_2PO_4, and 0.1% agar; pH 6.0). The flask was shaken on a rotary shaker at 27 °C for 3 days. The seed culture (1.0 mL) was transferred into a 500 mL Erlenmeyer flask ×48 containing 100 mL production medium (3.0% soluble starch, 1.0% glycerol, 2.0% soy bean meal, 0.3% dry yeast, 0.3% KCl, 0.2% $CaCO_3$, 0.05% $MgSO_4{\cdot}7H_2O$, and 0.1% KH_2PO_4, pH 6.5). Fermentation was carried out under shaking conditions at 27 °C. After 5 days of fermentation, the culture broth (4.8 L) was centrifuged at 8000 rpm for 10 min to collect the mycelia. The cell body was treated with acetone (2 L). After the acetone extracts were filtered and concentrated, they were applied to a Diaion HP-20 column (60 mL, Mitsubishi Chemical. Co., Tokyo, Japan). After washing with H_2O, active principles were eluted

DOI: 10.1021/acs.joc.6b00952
J. Org. Chem. 2016, 81, 7373−7383

with MeOH and concentrated under reduced pressure to give a black material (1.1 g). This material was applied to an octadecylsilyl (ODS) column (35 g, Fuji Silysia Chemical Ltd., Aichi, Japan) and eluted stepwise with 20, 40, 60, and 80% CH_3CN aqueous and 100% CH_3CN (140 mL each). The 80% CH_3CN fraction was collected and concentrated under reduced pressure to give brown materials containing **2** and **3** (83 mg). Some of the materials (83 mg dissolved in 3.3 mL MeOH) were finally purified by preparative HPLC under the following conditions: column, PEGASIL ODS SP100, 20 × 250 mm; mobile phase, a 30 min gradient from 40% CH_3CN-0.05% TFA to 70% CH_3CN-0.05% TFA, followed by 70% CH_3CN-0.05% TFA for 30 min; detection, ELSD with gas flow of 1.5 mL/min and tube temperature at 40 °C); flow rate, 8 mL/min. Under these conditions, **2** and **3** were eluted as peaks with retention times of 39 and 43 min, respectively. The fraction was concentrated under reduced pressure to give pure **2** (23.6 mg) and **3** (1.9 mg) as white powders.

*Valsafungin A (**2**).* $[\alpha]^{23}_{D}$ + 6.9 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3400, 2924, 2852, 2364, 1709 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRFABMS *m*/*z* 331.2845 $[M + H]^+$ (calcd for $C_{19}H_{39}O_4$ *m*/*z* 331.2848).

*Valsafungin B (**3**).* $[\alpha]^{23}_{D}$ + 29.1 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3422, 2924, 2855, 1708, 1647 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRFABMS *m*/*z* 329.2688 $[M + H]^+$ (calcd for $C_{19}H_{37}O_4$ *m*/*z* 329.2692).

Simplifungin Methyl Ester (4). A mixture of acetyl chloride (22.3 μL, 624 μmol) and MeOH (1.0 mL) was added to the powder of **1** (20.7 mg, 62.4 μmol), and the resulting solution was stirred at 0 °C for 1 min. The reaction mixture was stirred at 60 °C for 18 h under a N_2 atmosphere and concentrated in vacuo. The residue was dissolved in $CHCl_3$ and washed with saturated $NaHCO_3$ solution, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash silica gel column chromatography (1:20 MeOH/CH_2Cl_2) to afford the simplifungin methyl ester (**4**) (11.1 mg, 52%) as a white powder: $[\alpha]^{24}_{D}$ + 13.3 (*c* 0.1, $CHCl_3$); ^1H NMR (400 MHz, MeOH-d_4) δ 4.09 (1H, m), 3.98 (1H, d, *J* = 3.4), 3.85 (3H, s), 3.50 (1H, m), 1.51−1.59 (2H, m), 1.29−1.46 (24H, m), 0.91 (3H, t, *J* = 7.2); ^{13}C NMR (100 MHz, MeOH-d_4) δ 170.1, 72.4, 70.2, 58.8, 53.6, 38.5, 38.4, 34.9, 33.0, 30.8, 30.7, 30.6, 30.5, 30.4, 26.79, 26.78, 26.73, 23.7, 14.4; HRESI-MS *m*/*z* 368.2754 $[M + Na]^+$ (calcd for $C_{19}H_{39}NNaO_4$ *m*/*z* 368.2777).

Oxazolidinone Derivative 5. Di-*tert*-butyl dicarbonate (3.36 μL, 14.6 μmol) and 4-(dimethyamino)pyridine (DMAP) (1.70 mg, 13.9 μmol) were added to a solution of **2** (2.40 mg, 6.96 μmol) in CH_3CN (0.5 mL). After stirring at room temperature for 30 min, the reaction mixture was quenched with distilled water and diluted with EtOAc. The organic layer was washed with 1 N HCl and H_2O, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash silica gel column chromatography (1:2 EtOAc/Hexane) to afford **5** (1.80 mg, 55%) as a white powder: $[\alpha]^{24}_{D}$ + 7.5 (c 0.1, $CHCl_3$); ^1H NMR (600 MHz, $CDCl_3$) δ 4.36 (1H, d, *J* = 4.5), 4.33 (1H, ddd, *J* = 7.5, 5.5, 4.5), 3.82 (3H, s), 3.58 (1H, m), 1.80 (1H, m), 1.70 (1H, m), 1.51 (9H, s), 1.46 (1H, m), 1.43 (1H, m), 1.37−1.49 (6H, m), 1.29 (2H, m), 1.22−1.35 (14H, m), 0.89 (3H, t, *J* = 7.0); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.2, 150.7, 148.8, 84.6, 75.8, 72.0, 61.3, 53.0, 37.5, 37.4, 35.3, 31.8, 29.6, 29.38, 29.35, 29.2, 29.0, 27.8, 27.8, 27.8, 25.60, 25.58, 24.1, 22.6, 14.1; HRESI-MS *m*/*z* 494.3103 $[M + Na]^+$ (calcd for $C_{25}H_{45}NNaO_7$ *m*/*z* 494.3094).

***N*-Acetyl Simplifungin Methyl Ester (6).** Triethylamine (6.10 μL, 43.5 μmol) was added to a solution of **2** (7.50 mg, 21.7 μmol) in CH_2Cl_2 (1.0 mL), followed by acetyl chloride (1.55 μL, 21.7 μmol) dropwise at 0 °C. After stirring at 0 °C for 15 min, the reaction mixture was quenched with MeOH (1.0 mL) and concentrated in vacuo. The residue was purified by flash silica gel column chromatography (1:25 MeOH/CH_2Cl_2) to afford **6** (4.40 mg, 11.4 μmol, 53%) as a white powder: $[\alpha]^{26}_{D}$ + 13.1 (*c* 0.1, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 6.23 (1H, d, *J* = 9.0), 4.66 (1H, dd, *J* = 9.0, 2.0), 4.12 (1H, dt, *J* = 6.7, 2.0), 3.77 (3H, s), 3.58 (1H, m), 2.08 (3H, s), 1.77 (2H, m), 1.25−1.51 (24H, m), 0.88 (3H, t, *J* = 6.9); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.9, 171.5, 71.99, 71.94, 55.9, 52.6, 37.5, 37.4, 33.7, 31.8, 29.7, 29.6, 29.4, 29.33, 29.27, 25.63, 25.58, 25.49, 23.2, 22.6, 14.1; HRESI-MS *m*/*z* 410.2883 $[M + Na]^+$ (calcd for $C_{21}H_{41}NNaO_5$ *m*/*z* 410.2882).

(*S*)-MTPA Esters 7 and 8. EDCI·HCl (25.8 mg, 135 μmol), DMAP (4.8 mg, 39.3 μmol), and (*S*)-MTPA (26.4 mg, 113 μmol) were added to a solution of **6** (1.00 mg, 2.58 μmol) in CH_2Cl_2 (1.0 mL). After stirring at room temperature for 42 h, the reaction mixture was diluted with 1 N HCl and extracted with EtOAc. The organic layer was washed twice with 1 N HCl, saturated $NaHCO_3$ solution, and distilled water and then dried with Na_2SO_4 and concentrated in vacuo. The residue was purified by HPLC (column: SILICA, Shiseido; solvent: 97:3 hexane/2-propanol; detection: UV at 254 nm; flow rate: 1.0 mL/min) to give **7** (0.36 mg, 0.60 μmol, 23%) and **8** (0.28 mg, 0.34 μmol, 13%) as colorless oils.

*Compound **7**.* $[\alpha]^{23}_{D}$ −8.8 (*c* 0.06, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.49−7.52 (2H, m), 7.41−7.45 (3H, m), 5.75 (1H, d, *J* = 9.5), 5.55 (1H, t, *J* = 6.5), 4.93 (1H, d, *J* = 9.5), 3.69 (3H, s), 3.58 (1H, m), 3.51 (3H, s), 2.00 (3H, s), 1.71 (1H, m), 1.66 (1H, m), 1.23−1.60 (24H, m), 0.89 (3H, t, *J* = 6.5); HRESI-MS *m*/*z* 626.3279 $[M + Na]^+$ (calcd for $C_{31}H_{48}F_3NNaO_7$ *m*/*z* 626.3281).

*Compound **8**.* $[\alpha]^{24}_{D}$ + 46.0 (*c* 0.026, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.50−7.56 (4H, m), 7.37−7.45 (6H, m), 5.74 (1H, d, *J* = 9.3), 5.54 (1H, dt, *J* = 7.0, 2.0), 5.08 (1H, m), 4.92 (1H, dd, *J* = 9.3, 2.0), 3.69 (3H, s), 3.56 (3H, s), 3.51 (3H, s), 2.00 (3H, s), 1.50−1.71 (10H, m), 1.23−1.35 (10H, m), 1.23−1.35 (6H, m), 0.88 (3H, t, *J* = 7.0); HRESI-MS *m*/*z* 842.3689 $[M + Na]^+$ (calcd for $C_{41}H_{55}F_6NNaO_9$ *m*/*z* 842.3679).

(*R*)-MTPA Esters 9 and 10. EDCI·HCl (25.5 mg, 133 μmol), DMAP (4.00 mg, 32.7 μmol), and (*R*)-MTPA (25.4 mg, 109 μmol) were added to a solution of **6** (1.00 mg, 2.58 μmol) in CH_2Cl_2 (1.0 mL). After stirring at room temperature for 48 h, the reaction mixture was diluted with 1 N HCl and extracted with EtOAc. The organic layer was washed twice with 1 N HCl, saturated $NaHCO_3$ solution, and distilled water and then dried with Na_2SO_4 and concentrated in vacuo. The residue was purified by HPLC (column: SILICA, Shiseido; solvent: 97:3 hexane/2-propanol; detection: UV at 254 nm; flow rate: 1.0 mL/min) to give **9** (0.55 mg, 0.91 μmol, 35%) and **10** (1.05 mg, 1.28 μmol, 50%) as colorless oils.

*Compound **9**.* $[\alpha]^{24}_{D}$ + 0.7 (*c* 0.044, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.47−7.50 (2H, m), 7.40−7.44 (3H, m), 5.82 (1H, d, *J* = 9.5), 5.53 (1H, dt, *J* = 7.0, 2.0), 4.93 (1H, dd, *J* = 9.5, 2.0), 3.73 (3H, s), 3.58 (1H, m), 3.50 (3H, s), 2.02 (3H, s), 1.60 (1H, m), 1.56 (1H, m), 1.49−1.59 (5H, m), 1.35−1.49 (5H, m), 1.21−1.35 (14H, m), 0.89 (3H, t, *J* = 6.5); HRESI-MS *m*/*z* 626.3286 $[M + Na]^+$ (calcd for $C_{31}H_{48}F_3NNaO_7$ *m*/*z* 626.3281).

*Compound **10**.* $[\alpha]^{24}_{D}$ + 26.2 (*c* 0.1, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.54−7.56 (2H, m), 7.48−7.50 (2H, m), 7.37−7.44 (6H, m), 5.81 (1H, d, *J* = 9.4), 5.53 (1H, dt, *J* = 7.0, 2.3), 5.08 (1H, m), 4.93 (1H, dd, *J* = 9.4, 2.3), 3.73 (3H, s), 3.56 (3H, s), 3.50 (3H, s), 2.02 (3H, s), 1.51−1.67 (10H, m), 1.15−1.30 (16H, m), 0.86 (3H, t, *J* = 7.3); HRESI-MS *m*/*z* 842.3675 $[M + Na]^+$ (calcd for $C_{41}H_{55}F_6NNaO_9$ *m*/*z* 842.3679).

(*S*)-MTPA Amide 11. Pyridine (2.30 μL, 28.6 μmol) and (*R*)-MTPA chloride (0.58 μL, 3.10 μmol) were added to a solution of **4** (1.00 mg, 2.90 μmol) in $CHCl_3$ (1.0 mL). After stirring at room temperature for 4 h, the reaction mixture was concentrated in vacuo. The residue was purified by HPLC (column: PEGASIL ODS SP100; solvent: 30−90% CH_3CN (30 min gradient); detection: UV at 210 nm; flow rate: 1.0 mL/min) to give **11** (0.60 mg, 1.07 μmol, 38%) as a colorless oil: $[\alpha]^{23}_{D}$ −8.8 (*c* 0.06, MeOH); ^1H NMR (600 MHz, MeOH-d_4) δ 7.58−7.60 (2H, m), 7.40−7.45 (3H, m), 4.64 (1H, d, *J* = 2.5), 4.10 (1H, dt, *J* = 9.0, 2.5), 3.76 (3H, s), 3.65 (3H, d, 1.5 Hz), 3.51 (1H, m), 1.20−1.46 (26H, m), 0.91 (3H, t, 7.0 Hz) ; HRESI-MS *m*/*z* 584.3179 $[M + Na]^+$ (calcd for $C_{29}H_{46}F_3NNaO_6$ *m*/*z* 584.3175).

(*R*)-MTPA Amide 12. Pyridine (2.30 μL, 28.6 μmol) and (*S*)-MTPA chloride (0.58 μL, 3.10 μmol) were added to a solution of **4** (1.00 mg, 2.90 μmol) in $CHCl_3$ (1.0 mL). After stirring at room temperature for 40 min, the reaction mixture was concentrated in vacuo. The residue was purified by HPLC (column: PEGASIL ODS SP100; solvent: 30−90% CH_3CN (30 min gradient); detection: UV at 210 nm; flow rate: 1.0 mL/min) to give **12** (0.44 mg, 0.78 μmol, 27%) as a colorless oil: $[\alpha]^{24}_{D}$ + 0.7 (*c* 0.044, MeOH); ^1H NMR (600 MHz, MeOH-d_4) δ 7.72 (1H, dd, *J* = 3.7, 2.3), 7.62−7.63 (2H, m), 7.45−7.48 (2H, m), 4.60 (1H, d, *J* = 2.5),

DOI: 10.1021/acs.joc.6b00952
J. Org. Chem. 2016, 81, 7373−7383

4.18 (1H, ddd, J = 8.0, 6.0, 2.5), 3.76 (3H, s), 3.50 (1H, m), 3.38 (3H, s), 1.29−1.70 (26H, m), 0.87−0.92 (3H, m); HRESI-MS m/z 584.3180 $[M + Na]^+$ (calcd for $C_{29}H_{46}F_3NNaO_6$ m/z 584.3175).

(*S*)-2-NMA Ester 13. (*S*)-2-NMA (3.30 mg, 15.3 μmol), 1-ethyl-3-(3-dimethyaminopropyl)-carbodiimide hydrochloride (EDC·HCl) (5.20 mg, 27.1 μmol), and DMAP (3.00 mg, 24.6 μmol) were added to a solution of **5** (1.00 mg, 2.12 μmol) in CH_2Cl_2 (1.0 mL). After stirring at room temperature for 24 h, the reaction mixture was diluted with distilled water and extracted with $CHCl_3$. The organic layer was recovered and concentrated in vacuo to dryness. The residue was purified by HPLC (column: PEGASIL ODS SP100; solvent: 70−100% CH_3CN (30 min gradient); detection: UV at 210 nm; flow rate: 1.0 mL/min) to give **13** (0.44 mg, 0.66 μmol, 31%) as a colorless oil: $[\alpha]^{24}_D$ + 16.5 (c 0.1, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.92 (1H, s), 7.80−7.85 (3H, m), 7.56 (1H, dd, J = 8.5, 1.5), 7.47−7.50 (2H, m), 4.91 (1H, s), 4.91 (1H, m), 4.36 (1H, d, J = 4.5), 4.33 (1H, ddd, J = 7.5, 5.5, 4.5), 3.82 (3H, s), 3.46 (3H, s), 1.78 (1H, m), 1.72 (1H, m), 1.52 (9H, s), 1.42 (1H, m), 1.36 (1H, m), 1.19−1.58 (11H, m), 1.00 (2H, m), 0.81−1.19 (9H, m), 0.70 (3H, t, J = 7.5); HRESI-MS m/z 692.3783 $[M + Na]^+$ (calcd for $C_{38}H_{55}NNaO_9$ m/z 692.3775).

(*R*)-2-NMA Ester 14. (*R*)-2-NMA (3.20 mg, 14.8 μmol), EDC·HCl (4.70 mg, 24.5 μmol), and DMAP (2.60 mg, 21.3 μmol) were added to a solution of **5** (1.00 mg, 2.12 μmol) in CH_2Cl_2 (1.0 mL). After stirring at room temperature for 40 h, the reaction mixture was diluted with distilled water and extracted with $CHCl_3$. The organic layer was recovered and concentrated in vacuo to dryness. The residue was purified by HPLC (column: PEGASIL ODS SP100; solvent: 70−100% CH_3CN (30 min gradient); detection: UV at 210 nm; flow rate: 1.0 mL/min) to give **14** (0.41 mg, 0.61 μmol, 29%) as a colorless oil: $[\alpha]^{24}_D$ −4.3 (c 0.053, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.91 (1H, s), 7.81−7.85 (3H, m), 7.56 (1H, dd, J = 8.5, 1.5), 7.47−7.50 (2H, m), 4.91 (1H, s), 4.91 (1H, m), 4.35 (1H, d, J = 4.5), 4.31 (1H, ddd, J = 7.5, 5.5, 4.5), 3.82 (3H, s), 3.46 (3H, s), 1.74 (1H, m), 1.68 (1H, m), 1.52 (9H, s), 1.35 (1H, m), 1.29 (1H, m), 1.23 (2H, m), 1.10−1.58 (11H, m), 0.84 (3H, t, J = 7.5), 0.82−1.02 (9H, m); HRESI-MS m/z 692.3783 $[M + Na]^+$ (calcd for $C_{38}H_{55}NNaO_9$ m/z 692.3775).

Valsafungin A Methyl Ester (15). A mixture of acetyl chloride (63.7 μL, 70.1 μmol) and MeOH (1.0 mL) was added to the powder of **2** (11.8 mg, 35.7 μmol), and the resulting solution was stirred at 0 °C for 1 min. The reaction mixture was stirred at 60 °C for 18 h under a N_2 atmosphere and concentrated in vacuo. The residue was purified by flash silica gel column chromatography (1:50 MeOH/CH_2Cl_2) to afford the valsafungin A methyl ester (**15**) (8.90 mg, 73%) as a white powder: $[\alpha]^{24}_D$ + 10.6 (c 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, MeOH-d_4) δ 3.69 (3H, s), 3.69 (1H, dd, J = 10.7, 8.2), 3.61 (1H, dd, J = 10.7, 5.5), 3.50 (1H, m), 2.55 (1H, ddt, J = 8.6, 8.2, 5.5), 1.23−1.58 (28H, m), 0.90 (3H, t, J = 7.0); ^{13}C NMR (100 MHz, MeOH-d_4) δ 177.1, 72.4, 64.2, 52.0, 49.9, 38.45, 38.42, 33.1, 30.8, 30.7, 30.61, 30.58, 30.55, 30.50, 29.7, 28.4, 26.8, 26.8, 23.7, 14.4; HRESI-MS m/z 367.2822 (calcd for $C_{20}H_{40}NaO_4$ m/z 367.2824).

(*S*)-9-AMA Ester 16. (*S*)-9-AMA (2.0 mg, 13.6 μmol), EDC·HCl (2.70 mg, 14.1 μmol), and DMAP (cat.) were added to a solution of **15** (1.00 mg, 2.91 μmol) in CH_2Cl_2 (1.0 mL). After stirring at room temperature for 36 h, the reaction mixture was diluted with distilled water and extracted with CH_2Cl_2. The organic layer was washed with 1 N HCl, saturated $NaHCO_3$ solution, and distilled water and then dried with Na_2SO_4 and concentrated in vacuo. The residue was purified by HPLC (column: SILICA; solvent: 99:1 hexane/2-propanol; detection: UV at 260 nm; flow rate: 1.0 mL/min) to give **16** (0.24 mg, 0.41 μmol, 14%) as a colorless oil: $[\alpha]^{24}_D$ + 60.1 (c 0.015, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (2H, d, J = 8.5), 8.48 (1H, s), 8.02 (2H, d, J = 8.5), 7.57−7.46 (4H, m), 6.25 (1H, s), 4.20 (1H, dd, J = 11.0, 6.0), 4.17 (1H, dd, J = 11.0, 8.0), 3.58 (1H, m), 3.41 (3H, s), 3.18 (3H, s), 2.32 (1H, tt, J = 8.0, 6.0), 1.56−0.79 (26H, m), 0.99 (2H, m), 0.88 (3H, t, J = 7.5); HRESI-MS m/z 615.3641 (calcd for $C_{37}H_{52}NaO_6$ m/z 615.3662).

(*R*)-9-AMA Ester 17. (*R*)-9-AMA (2.00 mg, 13.6 μmol), EDC·HCl (2.70 mg, 14.1 μmol), and DMAP (cat.) were added to a solution of **15** (1.00 mg, 2.91 μmol) in CH_2Cl_2 (1.0 mL). After stirring at room temperature for 42 h, the reaction mixture was diluted with distilled water and extracted with CH_2Cl_2. The organic layer was washed with 1 N HCl, saturated $NaHCO_3$ solution, and distilled water and then dried with Na_2SO_4 and concentrated in vacuo. The residue was purified by HPLC (column: SILICA; solvent: 98.5:1.5 hexane/2-propanol; detection: UV at 260 nm; flow rate: 1.0 mL/min) to give **17** (0.23 mg, 0.39 μmol, 13%) as a colorless oil: $[\alpha]^{24}_D$ + 72.0 (c 0.014, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (2H, d, J = 8.5), 8.46 (1H, s), 8.00 (2H, d, J = 8.5), 7.55−7.44 (4H, m), 6.24 (1H, s), 4.15 (1H, dd, J = 10.5, 5.5), 4.11 (1H, dd, J = 10.5, 7.0), 3.58 (1H, m), 3.41 (3H, s), 3.19 (3H, s), 2.41 (1H, tt, J = 7.0, 5.5), 1.56−0.78 (26H, m), 0.94 (2H, m), 0.87 (3H, t, J = 7.5); HRESI-MS m/z 615.3645 (calcd for $C_{37}H_{52}NaO_6$ m/z 615.3662).

Benzoyl Derivative 18. Benzoic acid (1.70 mg, 13.9 μmol), EDC·HCl (3.30 mg, 17.4 μmol), and DMAP (cat.) were added to a solution of **15** (4.00 mg, 8.93 μmol) in CH_2Cl_2 (0.5 mL). After stirring at 0 °C for 30 min, the reaction mixture was stirred at room temperature for 4 h, diluted with 1 N HCl, and extracted with EtOAc. The organic layer was washed twice with distilled water and then dried with Na_2SO_4 and concentrated in vacuo. The residue was subjected to preparative silica gel TLC developed by 4:1 hexane/EtOAc and the silica gel on a band (R_f ≈ 0.76) was harvested. The substances adsorbed by silica gels were then eluted by EtOAc to give **18** (2.90 mg, 6.47 μmol, 56%) as a white powder: $[\alpha]^{24}_D$ −1.8 (c 0.07, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (2H, d, J = 7.4), 7.56 (1H, t, J = 7.4), 7.43 (2H, t, J = 7.4), 4.47 (1H, dd, J = 11.0, 5.5), 4.43 (1H, dd, J = 11.0, 7.8), 3.72 (3H, s), 3.58 (1H, m), 2.86 (1H, tt, J = 7.8, 5.5), 1.72 (1H, m), 1.26−1.64 (27H, m), 0.88 (3H, t, J = 7.0); ^{13}C NMR (150 MHz, $CDCl_3$) δ 174.2, 166.2, 133.1, 129.95, 129.62, 129.62, 128.4, 128.4, 72.0, 65.1, 51.9, 45.0, 37.52, 37.50, 31.9, 29.7, 29.6, 29.45, 29.41, 29.39, 29.34, 28.8, 27.0, 25.6, 25.6, 22.6, 14.1; HRESI-MS m/z 471.3090 $[M + Na]^+$ (calcd for $C_{27}H_{44}NaO_5$ m/z 471.3086).

(*S*)-2-NMA Ester 19. (*S*)-2-NMA (2.90 mg, 13.4 μmol), EDC·HCl (5.10 mg, 26.6 μmol), and DMAP (2.50 mg, 20.5 μmol) were added to a solution of **18** (1.00 mg, 2.23 μmol) in CH_2Cl_2 (1.0 mL). After stirring at room temperature for 116 h, the reaction mixture was diluted with 1 N HCl and extracted with EtOAc. The organic layer was washed twice with 1 N HCl and distilled water and then dried with Na_2SO_4 and concentrated in vacuo. The residue was purified by HPLC (column: SILICA; solvent: 99.7:0.3 hexane/2-propanol; detection: UV at 254 nm; flow rate: 1.0 mL/min) to give **19** (0.72 mg, 1.11 μmol, 50%) as a colorless oil: $[\alpha]^{24}_D$ + 36.4 (c 0.072, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (2H, d, J = 8.5), 7.92 (1H, s), 7.86−7.79 (3H, m), 7.58−7.54 (2H, m), 7.50−7.41 (4H, m), 4.90 (1H, s), 4.90 (1H, m), 4.47 (1H, dd, J = 11.0, 6.0), 4.43 (1H, dd, J = 11.0, 8.0), 3.72 (3H, s), 3.46 (3H, s), 2.87 (1H, tt, J = 8.0, 6.0), 1.72 (1H, m), 1.60 (1H, m), 1.56−0.79 (16H, m), 1.33 (2H, m), 1.25 (2H, m), 1.20 (2H, m), 0.98 (2H, m), 0.84 (2H, m), 0.70 (3H, t, J = 7.5); HRESI-MS m/z 669.3758 (calcd for $C_{40}H_{54}NaO_7$ m/z 669.3767).

(*R*)-2-NMA Ester 20. (*R*)-2-NMA (5.80 mg, 26.8 μmol), EDC·HCl (10.30 mg, 53.7 μmol), and DMAP (4.90 mg, 40.1 μmol) were added to a solution of **18** (1.00 mg, 2.23 μmol) in CH_2Cl_2 (1.0 mL). After stirring at room temperature for 44 h, the reaction mixture was diluted with 1 N HCl and extracted with EtOAc. The organic layer was washed twice with 1 N HCl distilled water and then dried with Na_2SO_4 and concentrated in vacuo. The residue was purified by HPLC (column: SILICA; solvent: 95:5 hexane/EtOAc; detection: UV at 260 nm; flow rate: 1.0 mL/min) to give **20** (0.52 mg, 0.80 μmol, 36%) as a colorless oil: $[\alpha]^{24}_D$ −28.4 (c 0.052, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (2H, d, J = 8.5), 7.92 (1H, s), 7.86−7.79 (3H, m), 7.58−7.54 (2H, m), 7.50−7.41 (4H, m), 4.90 (1H, s), 4.90 (1H, m), 4.47 (1H, dd, J = 11.0, 6.0), 4.43 (1H, dd, J = 11.0, 8.0), 3.72 (3H, s), 3.46 (3H, s), 2.86 (1H, tt, J = 8.0, 6.0), 1.70 (1H, m), 1.58 (1H, m), 1.55−0.80 (20H, m), 1.30 (2H, m), 1.22 (2H, m), 1.09 (2H, m), 0.84 (3H, t, J = 7.5); HRESI-MS m/z 669.3759 (calcd for $C_{40}H_{54}NaO_7$ m/z 669.3767).

Preparation of Ketodihydrosphingosine (24). *tert-Butyl (S)-(3-hydroxy-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)carbamate (**22**).* A solution of *N*-Boc-L-serine (**21**) (1.50 g, 7.31 mmol) in CH_2Cl_2 (15.0 mL) was treated with *N*,*O*-dimethylhydroxylamine hydrochloride (784 mg, 8.04 mmol) and *N*-methylmorpholine (804 μL, 8.04 mmol) at −15 °C. After stirring at −15 °C for 5 min, EDCI (2.10 g, 11.0 mmol) was added to the mixture. After stirring at −15 °C for 1 h, the reaction

DOI: 10.1021/acs.joc.6b00952
J. Org. Chem. 2016, 81, 7373−7383

mixture was quenched with 1 N HCl. The organic layer was washed with H_2O, dried over Na_2SO_4, filtered, and concentrated in vacuo to afford **22** (1.50 g, 82%) as a white solid: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.58 (1H, brs), 4.80 (1H, brs), 3.82 (2H, dd, J = 4.0, 4.4 Hz), 3.78 (3H, s), 3.24 (3H, s), 1.49 (9H, s).

(Literature data[17]); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.71 (1H, brs), 4.95 (1H, brs), 3.82−3.78 (5H, m), 3.23 (3H, s), 2.90 (1H, brs), 1.43 (9H, s).

*tert-Butyl (S)-(1-hydroxy-3-oxooctadecan-2-yl)carbamate (**23**).* A solution of **22** (1.00 g, 4.03 mmol) in THF (8.0 mL) was treated with *s*-BuLi (2.0 M sol. in Et_2O, 4.03 mL, 8.06 mmol) at −15 °C. After stirring at −15 °C for 5 min, *n*-$C_{15}H_{31}MgBr$ (2.0 M sol. in Et_2O, 9.70 mL, 4.83 mmol) was added to the mixture. After stirring at room temperature for 4 h, the reaction mixture was quenched with 1 N HCl and diluted with EtOAc. The organic layer was washed with H_2O, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash silica gel column chromatography (0.75:100 MeOH/CH_2Cl_2) to afford **23** (1.31 g, 81%) as a white solid: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.37 (1H, brs), 4.07 (1H, brs), 3.93 (2H, dd, J = 2.0, 3.2 Hz), 2.55 (2H, q, J = 8.0 Hz), 1.61−1.58 (2H, m), 1.49 (9H, s), 1.29−1.22 (26H, m), 0.87 (3H, t, J = 6.8 Hz).

(Literature data[17]); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.63 (1H, brs), 4.34 (1H, s), 3.90−3.97 (2H, m), 2.63 (1H, brs), 2.48−2.63 (2H, m), 1.57 (2H, m), 1.45 (9H, s), 1.22−1.27 (24H, m), 0.88 (3H, t, J = 6.8 Hz).

*Ketodihydrosphingosine (**24**).* A solution of **23** (912 mg, 2.28 mmol) in MeOH (10.0 mL) was treated with AcCl (1.63 mL, 22.8 mmol) at 0 °C.[17] After stirring at room temperature for 0.5 h, the white solid was filtered off, washed with EtOAc, and dried to afford ketodihydrosphingosine (395 mg, 58%) as a white solid. The mother liquor was concentrated in vacuo. The residue was purified by flash silica gel column chromatography (7:100−10:100 MeOH/CH_2Cl_2) to afford **24** (131 mg, 19%, total 77%) as a white solid: $[\alpha]^{24}_D$ + 27.8 (c 1.0, $CHCl_3$); IR (neat) 3436, 3005, 2921, 1659, 1436, 1408, 1316, 1046, 1027 cm^{-1}; ^{1}H NMR (MeOH-d_4, 400 MHz) δ 4.14 (1H, t, J = 3.6 Hz), 3.96 (1H, dd, J = 3.6, 12.4 Hz), 3.76 (1H, dd, J = 3.6, 12.4 Hz), 2.59−2.55 (2H, m), 1.47 (1H, brt, J = 6.0 Hz), 1.27−1.18 (26H, m), 0.83 (t, 3H, CH_3, J = 6.8 Hz); ^{13}C NMR (MeOH-d_4, 100 MHz) δ 203.8, 60.8, 58.9, 38.2, 31.7, 29.4, 29.4, 29.3, 29.2, 29.1, 29.0, 28.7, 22.8, 22.3, 13.0; HRESI-MS m/z 322.2718 $[M + Na]^+$ (calcd for $C_{18}H_{37}NNaO_2$ m/z 322.2722).

Assay for Antifungal Activity. Agar diffusion method using paper disks (Method A): *C. albicans* ATCC 64548 was inoculated into a 50 mL test tube containing 10 mL of Waksman broth (2.0% glucose, 1.0% meat extract, 0.5% peptone, 0.3% dry yeast, 0.5% NaCl, and 0.3% $CaCO_3$, pH 7.0) and was grown for 48 h on the reciprocal shaker. The seed culture was transferred to GY agar (1.0% glucose, 0.5% yeast extract and 0.8% agar, pH 6.0). A paper disk (6 mm, ADVANTEC, Tokyo, Japan) containing the test sample was placed on the agar plate, which was then incubated at 27 °C for 24 h. Activities were expressed as the diameter (mm) of the inhibitory zone.

Broth microdilution method (Method B): Five yeast and eight fungal strains (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, *Aspergillus fumigatus* var. fumigatus, *A. flavas*, *A. niger*, *A. terreus*, *Rhizopus oryzae*, *R. microspores* var. rhizopodiformis, *Rhizomucor pusillus*, and *Absidia corymbifera*) were used for antifungal activity assays. *A. fumigatus* var. fumigatus, *A. flavas*, *A. niger*, *A. terreus*, *R. oryzae*, and *R. pusillus* were obtained from the NITE Biological Resource Center (Tokyo, Japan). *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. neoformans* were purchased from ATCC (Virginia, USA). *R. microspores* var. rhizopodiformis and *A. corymbifera* were obtained through the National BioResource Project in Japan. The broth microdilution method using 96-well microplates was performed according to the guidelines of the NCCLS documents M27-A3 and M38-A2.[11] Regarding yeast, five colonies with diameters of 1 mm were suspended in sterile 0.85% saline to adjust to a 0.5 McFarland standard by spectrophotometric measurements. The seed of yeast was diluted 2000 times with RPMI1640 medium (165 mM morpholinopropanesulfonic acid buffer (pH 7.0)). The diluted seed (180 μL) and RPMI1640 medium (20 μL) were added to each well of a 96-well microplate with or without serial concentrations of test compounds. Regarding filamentous fungi, spores were suspended in sterile 0.85% saline. After allowing heavy particles to settle for 5 min, the supernatant was transferred to a sterile tube and adjusted to an optical density at 550 nm (OD_{550}) that was 0.0621 for Aspergillus spp. or 0.0765 for Zygomycetous. This seed was diluted 50 times with RPMI1640 medium. The diluted seed (100 μL) and RPMI1640 medium (100 μL) were added to each well of a 96-well microplate with or without serial concentrations of test compounds. These 96-well plates were incubated at 35 °C for 24 h (*Candida* spp. and Zygomycetous), 48 h (*Aspergillus* spp.), or 72 h (*C. neoformans*). After the incubation, OD_{550} were measured to determine the minimum inhibitory concentration (MIC). The antifungal activities of test compounds were tested at concentrations ranging from 32 to 0.0625 μg/mL for **1** to **6**, **15** and **18**, and **16** to 0.0313 μg/mL for myriocin. The MIC was defined as the lowest concentration of the antifungal agent at which growth was inhibited by 50% from that of growth in the control.

Assay for SPT Activity. The microsome fractions prepared from *S. cerevisiae* S208C[18] were used in the SPT assay. *S. cerevisiae* was grown in YPD medium (2.0% polypeptone, 1.0% yeast extract, 2.0% glucose) at 27 °C for 24 h. Fresh cells were washed twice with 10 volumes of cold, distilled water, then suspended in 2 volumes of buffer A (potassium phosphate buffer (0.05 M, pH 7.0) containing DL-dithiothreitol (1 mM), phenylmethylsulfonyl fluoride (1 mM), and complete mini (1 tablet/10 mL)) and then disrupted in a French pressure cell press at 20000 psi. Intact cells, debris, and the mitochondrial fraction were sedimented by centrifugation at 14500*g* for 30 min. The supernatant solution was centrifuged at 49000 rpm for 90 min in a Beckman optima centrifuge at 4 °C to precipitate the microsome fraction containing SPT. The pellet was suspended in buffer B (potassium phosphate buffer (0.05 M, pH 7.0) containing DL-dithiothreitol (1 mM) and pyridoxal 5′-phosphate hydrate (0.1 mM)). The final protein concentration was approximately 30 mg/mL.

The assay conditions of SPT were set according to the method of Lester et al.[13] The reaction mixture contained triethanolamine (0.1 M, pH7.5), dithiothreitol (5 mM), EDTA-4Na (2.5 mM), pyridoxal 5′-phosphate hydrate (50 μM), L-serine (25 mM), palmitoyl-CoA (200 μM), and the microsome fraction prepared from *S. cerevisiae* (100 μg) in a final volume of 100 μL. After 20 min at 30 °C, the reaction was stopped by the addition of 0.5N NH_4OH (0.2 mL). The product was extracted from the reaction mixture by the modified Bligh and Dyer's method.[19] The $CHCl_3$ layer containing the reaction product KDS was dried and resuspended in 10 μL MeOH, which was used to quantify the amount of KDS by LC/ESI-MS under the following conditions: column, PEGASIL C8 SP100, 2.0 × 100 mm (Senshu Scientific Co., Tokyo, Japan); mobile phase, a 30 min gradient from 60% MeOH-0.1% HCO_2H to 70% MeOH-0.1% HCO_2H; column temperature, 40 °C; flow rate, 0.2 mL/min.

Assay for Antimicrobial Activity. Antimicrobial activity against the following six microorganisms was measured using the paper disk method.[20] *B. subtilis* PCI219, *S. aureus* FDA209P, *M. luteus* KB212, *E. coli* JM109, and *P. aeruginosa* IFO12689 were used for the assay.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b00952.

Taxonomic studies on *Valsaceae* sp. FKH-53, LC−MS data of the SPT assay for **2**, the structure of myriocin, biosynthetic pathway of sphingolipids, NMR spectra of **1** to **18** and **24**, and MS data of **1** to **3**. (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: tomodah@pharm.kitasato-u.ac.jp.

Notes

The authors declare no competing financial interest.

DOI: 10.1021/acs.joc.6b00952
J. Org. Chem. 2016, 81, 7373−7383

■ ACKNOWLEDGMENTS

We thank Ms. Noriko Sato (School of Pharmaceutical Sciences, Kitasato University) for the NMR spectral measurements. This work was supported by JSPS KAKENHI Grant Numbers 16H05095, 25460130, and 21310146.

■ REFERENCES

(1) Kontoyiannis, D. P. *Am. J. Med.* **2011**, *125*, S25−38.
(2) Castelli, M. V.; Butassi, E.; Monteiro, M. C.; Svetaz, L. A.; Vicente, F.; Zacchino, S. A. *Expert Opin. Ther. Pat.* **2014**, *24*, 323−338.
(3) Mathew, B. P.; Nath, M. *ChemMedChem* **2009**, *4*, 310−323.
(4) Knölker, H.-J.; Braxmeler, T. *Tetrahedron Lett.* **1998**, *39*, 9407−9410.
(5) Enders, D.; Kallfass, U.; Nolte, B. *Synlett* **2002**, *2002*, 33−36.
(6) Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092−4096.
(7) Dasyam, N.; Munkacsi, A. B.; Fadzilah, N. H.; Senanayake, D. S.; O'Toole, R. F.; Keyzers, R. A. *J. Nat. Prod.* **2014**, *77*, 1519−1523.
(8) Kusumi, T.; Fukushima, T.; Ohtani, I.; Kakisawa, H. *Tetrahedron Lett.* **1991**, *32*, 2939−2942.
(9) (a) Kusumi, T.; Takahashi, H.; Xu, P.; Fukushima, T.; Asakawa, Y.; Hashimoto, T.; Kan, Y.; Inouye, Y. *Tetrahedron Lett.* **1994**, *35*, 4397−4400. (b) Ishii, H.; Krane, S.; Itagaki, Y.; Berova, N.; Nakanishi, K.; Weldon, P. J. *J. Nat. Prod.* **2004**, *67*, 1426−1430.
(10) (a) Ferreiro, M. J.; Latypov, Sh. K.; Quiñoá, E.; Riguera, R. *Tetrahedron: Asymmetry* **1996**, *7*, 2195−2198. (b) Freire, F.; Seco, J. M.; Quiñoá, E.; Riguera, R. *Chem. Commun.* **2007**, 1456−1458. (c) Gutiérrerz, M.; Pereira, A. R.; Debonsi, H. M.; Ligresti, A.; Marzo, V.; Di Gerwick, W. H. *J. Nat. Prod.* **2011**, *74*, 2313−2317.
(11) (a) Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, Approved Standard (CLSI document M27-A3)*, 3rd ed.; Clinical and Laboratory Standards Institute; NCCLS: Wayne, PA, 2008; Vol. *28*, pp 1−25. (b) Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi, Approved Standard (CLSI document M38-A2)*, 2nd ed.; Clinical and Laboratory Standards Institute; NCCLS: Wayne, PA, 2008; Vol. *28*, pp 1−35.
(12) (a) Miyake, Y.; Kozutsumi, Y.; Nakamura, S.; Fujita, T.; Kawasaki, T. *Biochem. Biophys. Res. Commun.* **1995**, *211*, 396−403. (b) Kluepfel, D.; Bagli, J.; Baker, H.; Charest, M.-P.; Kudelski, A.; Sehgal, S. N.; Vézina, C. *J. Antibiot.* **1972**, *25*, 109−115.
(13) Lester, R. L.; Withers, B. R.; Schultz, M. A.; Dickson, R. C. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* **2013**, *1831*, 726−736.
(14) Sun, Y.; Taniguchi, R.; Tanoue, D.; Yamaji, T.; Takematsu, H.; Mori, K.; Fujita, T.; Kawasaki, T.; Kozutsumi, Y. *Mol. Cell. Biol.* **2000**, *20*, 4411−4419.
(15) Freire, F.; Seco, J. M.; Quiñoá, E.; Riguera, R. *Org. Lett.* **2010**, *12*, 208−211.
(16) Nonaka, K.; Kaifuchi, S.; Ōmura, S.; Masuma, R. *Mycoscience* **2013**, *54*, 42−53.
(17) So, R. C.; Ndonye, R.; Izmirian, D. P.; Richardson, S. K.; Guerrera, R. L.; Howell, A. R. *J. Org. Chem.* **2004**, *69*, 3233−3235.
(18) Braun, P. E.; Snell, E. E. *Proc. Natl. Acad. Sci. U. S. A.* **1967**, *58*, 298−303.
(19) Bligh, E. G.; Dyer, W. J. *Can. J. Biochem. Physiol.* **1959**, *37*, 911−917.
(20) Kobayashi, K.; Tsukasaki, N.; Uchida, R.; Yamaguchi, Y.; Tomoda, H. *J. Antibiot.* **2015**, *68*, 615−619.

DOI: 10.1021/acs.joc.6b00952
J. Org. Chem. 2016, 81, 7373−7383