A One-Pot Chemoenzymatic Synthesis of (2*S*,4*R*)-4-Methylproline Enables the First Total Synthesis of Antiviral Lipopeptide Cavinafungin B

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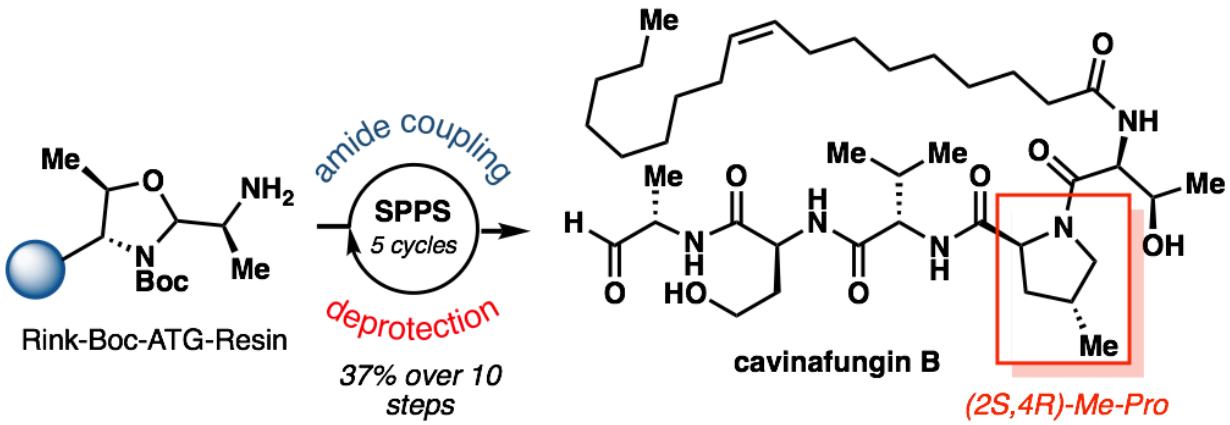
Abstract

We report an efficient ten-step (longest linear sequence) synthesis of antiviral natural product cavinafungin B in 37% overall yield. By leveraging a one-pot chemoenzymatic synthesis of (2*S*,4*R*)-4-methylproline and oxazolidine-tethered (Rink-Boc-ATG-Resin) SPPS methodology, the assembly of our molecular target could be conducted in an efficient manner. This general strategy could prove amenable to the construction of other natural and unnatural linear lipopeptides. The value of incorporating biocatalytic steps in complex molecule synthesis is highlighted by this work.

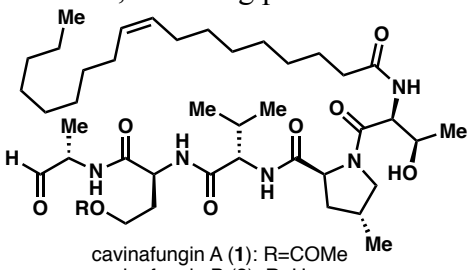


Keywords: biocatalysis, lipopeptide, chemoenzymatic synthesis, hydroxylation

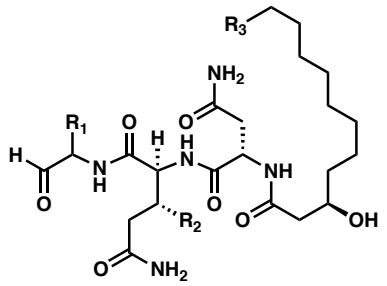
1. Introduction

The aldehyde lipopeptides cavinafungin A (**1**) and B (**2**) were originally isolated in 2015 as minor constituents of crude extracts from *Colispora cavincola*, collected during a bioactivity guided screen for potentiation of antifungal agent caspofungin.[1] Although the sought-after potentiation activity was eventually attributed to the depsipeptide colisporifungin, the cavinafungins demonstrated broad-spectrum antifungal properties (MIC of 0.4–4 μg/mL against *Candida* species and 8 μg/mL against *A. fumigatus*). Cavinafungin A and B are nearly identical lipopentapeptide aldehydes, differing only by acetylation at L-homoserine (Figure 1). The cavinafungins contain three noncanonical amino acids (L-alaninal, L-homoserine, and (2*S*,4*R*)-4-methylproline [(2*S*,4*R*)-4-MePro]), as well as an N-terminal oleamide lipid tail. A related family

of C-terminal peptide aldehydes called the fellutamides A–D (**3**–**6**) (Figure 1) display a range of activities, including proteasome inhibition and nerve growth factor synthesis induction.[2]



cavinafungin A (**1**): R=COMe
cavinafungin B (**2**): R=H



fellutamide A (**3**): R_1=^{i}Bu, R_2=OH, R_3=Me
fellutamide B (**4**): R_1=^{i}Bu, R_2=H, R_3=Me
fellutamide C (**5**): R_1=^{i}Pr, R_2=OH, R_3=Pr
fellutamide D (**6**): R_1=^{i}Bu, R_2=OH, R_3=Pr

Figure 1. The cavinafungins and related aldehyde lipopeptides.

More recently, cavinafungin A (**1**) was identified to be potently active against all four dengue virus serotypes (IC_{50}=1–5 nM, >100-fold selectivity over uninfected cells) and the Zika virus (IC_{50}=150 nM, *ca.* 30-fold selectivity over uninfected cells).[3] Aldehyde reduction to alcohol (cavinafungol) neutralized both antifungal and antiviral properties, suggesting the aldehyde is structurally important for biological activity.[1,3] A novel CRISPR/Cas9 protocol for genome-wide profiling in HCT116 cells identified the endoplasmic-reticulum-associated signal peptidase (ER-associated SPase) as the efficacy target of **1**.[4] SPase is an essential membrane-bound serine protease involved in cleaving signal peptides of secretory and membrane proteins in the ER. Mechanistically, **1** is proposed to anchor to the ER membrane through its lipophilic tail, followed by subsequent binding of the oligopeptide motif to the SEC11A peptide binding cleft of SPase. A proximal catalytic serine residue likely attacks the C-terminal aldehyde of cavinafungin A, resulting in the formation of a hemiacetal intermediate and covalent inhibition of SPase activity.[3] This proposed mechanism draws parallels to the classical serine protease inhibition mechanism.[5] Cavinafungin A was subsequently used to confirm that cleavage by SPase is essential for dengue virus protein processing, indicating that cavinafungin A would likely inhibit other homologous flavivirus possessing similar infection mechanism.[3] As cavinafungin A (**1**) and B (**2**) share identical antifungal activity, it is plausible that they also

display identical antiviral properties. Hypothetically, cavinafungin A may give rise to B *in vivo* upon acetyl hydrolysis, explaining their identical antifungal activities. Dengue and Zika viruses have no FDA-approved vaccination or specific antiviral treatment and cavinafungin is the only known selective eukaryotic SPase inhibitor. Thus, obtaining ample quantities of material for further study and application is of significant value.[3,6] The establishment of a robust synthetic strategy would also enable "Hit-to-Lead" optimization starting from cavinafungin B.

Recently, peptide-based drugs (~2–50 amino acids) have experienced a renaissance in the pharmaceutical industry due to their ability to bridge the beneficial properties of biological and small molecule therapeutics.[7] Advances in high-throughput sequencing technologies, bioinformatics-based genome mining, and combinatorial biosynthesis have catalyzed their re-entry into drug discovery programs.[7a,8] Although peptides modulate a suite of useful biological activities, they routinely display poor pharmacokinetic/dynamic properties, such as membrane impermeability and lability to peptidases, resulting in biological instability.[7] In contrast, natural and artificial lipopeptides can remedy some of these undesired properties.[7,9] Examples of FDA-approved lipopeptides for medical use include the antifungal micafungin and the antibiotics polymyxin B and daptomycin.[7b,9,10]

To attenuate the current supply demand for flaviviral probes and drug candidates, we targeted synthetic access to the cavinafungins. This communication documents our synthesis of cavinafungin B in 10 steps from known intermediates. Of note, we developed a one-pot chemoenzymatic synthesis of (2*S*,4*R*)-4-MePro which expedites the assembly of **2**.[11] Furthermore, our strategy delineates a protocol for constructing linear lipopeptides using fluorenylmethyloxycarbonyl (Fmoc) based solid-phase peptide synthesis (SPPS), opening the door towards analogs derived from cavinafungin's core structure.

2. Results and discussion

At the outset of our synthetic planning, we identified the C-terminal aldehyde, the (*2S*,*4R*)-4-MePro residue, and the oleamide motif as key synthetic challenges. Peptide aldehydes have diverse applications in chemical ligation, high-throughput protein profiling, peptide macrocyclization, and protease inhibition.[12] Several methods are known for introducing aldehydes into a peptide of interest, including alcohol oxidation, reduction of activated amides/esters/semicarbazones, and aldehyde protection via oxazolidine/thiazolidine heterocycles.[12a] Unfortunately, peptide aldehydes are prone to epimerization on isolation,[2] though this feature appears substrate dependent. Our synthetic approach was inspired by a recent report published by Baran and co-workers on a general solid-phase peptide synthesis (SPPS) strategy towards peptide aldehyde synthesis and macrocyclization, which resulted in the synthesis of 28 natural and unnatural peptide aldehydes (Figure 2).[12b] Briefly, the strategy

involved a *tert*-butoxycarbonyl (Boc) protected oxazolidine tether constructed from a suitable Fmoc-amino aldehyde and threonine–glycine (TG)-functionalized Rink amide resin. After iterative amide couplings and a final Fmoc deprotection, the resulting peptide could be easily cleaved from the TG-Rink amide resin, affording the desired peptide aldehyde after HPLC purification. Subsequently, suitable nucleophiles were used to capture the transient iminium species to yield the corresponding macrocyclic peptides.

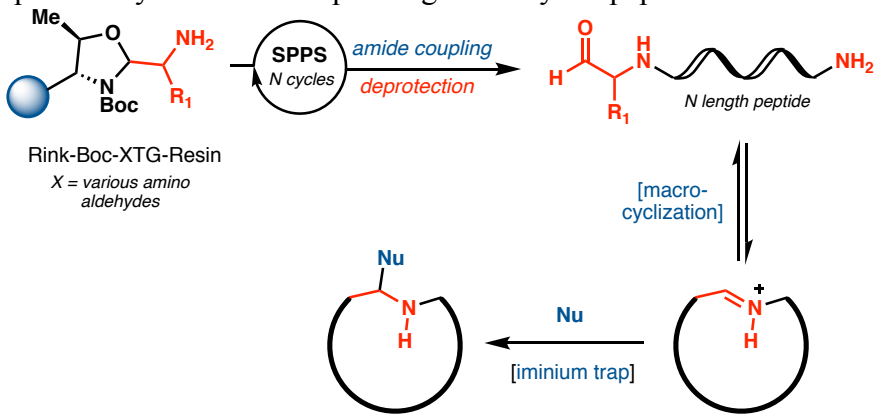


Figure 2. Previous application of Rink-Boc-XTG-resin SPPS in peptide aldehyde synthesis and subsequent macrocyclization.

This protocol proved applicable to cavinafungin B (**2**), starting from known Boc–alaninal–oxazolidine–threonine–glycine Rink amide resin (Rink-Boc-ATG-Resin) (Figure 3). Taking notes from the previously reported protocol, PyAOP and NMM were selected for amide coupling steps, and a solution of 20% piperidine/DMF was used for Fmoc deprotection steps (Figure 3).[12b] Although fragments **11**, **14**, and **16** are commercially available, **8** and **9** required preparation. The first fragment, **9,** was initially targeted as the *O*-trityl ether, but due to synthetic difficulties, the secondary alcohol was instead protected as the *tert*-butyldimethylsilyl (TBS) ether. Although (2*S*,4*R*)-4-MePro is present in a suite of biologically relevant natural peptides, it has proven nontrivial to construct in the literature.[13] Previous reports require the use of dangerous/expensive reagents (MeOTf/LiHMDS or Crabtree's Catalyst), directing groups, several protecting groups/functional group interconversions, and do not display complete stereocontrol.[13c,d] Recently, we reported a step efficient and high yielding one-pot chemoenzymatic synthesis of (2*S*,4*R*)-4-MePro from L-leucine (**7**).[11] Our protocol leverages the iron- and α-ketoglutarate dependent dioxygenase (Fe/αKG) GriE to perform C–H-oxygenation at the δ-position of **7** (Figure 3).[14] The resulting aminoaldehyde spontaneously cyclizes to an imine, and can be efficiently reduced to the secondary amine upon addition of NH_3•BH_3, resulting in yields as high as 88%.[15] Fmoc protection of (2*S*,4*R*)-4-MePro yielded **8** in 57% over two steps. Here, this sequence was carried out on 100 mg scale to provide adequate supply of material for

SPPS. According to previous reports, peptide lipidation via SPPS is commonly performed, although a general procedure for coupling oleic acid via SPPS could not be found.[16] In turn we adjusted known conditions (EDC/HOBt/DIPEA) reported by Zeng *et al*. for solution phase oleamide synthesis.[16b] Following global deprotection and cleavage from the resin, cavinafungin B was isolated in 37% overall yield after HPLC purification.

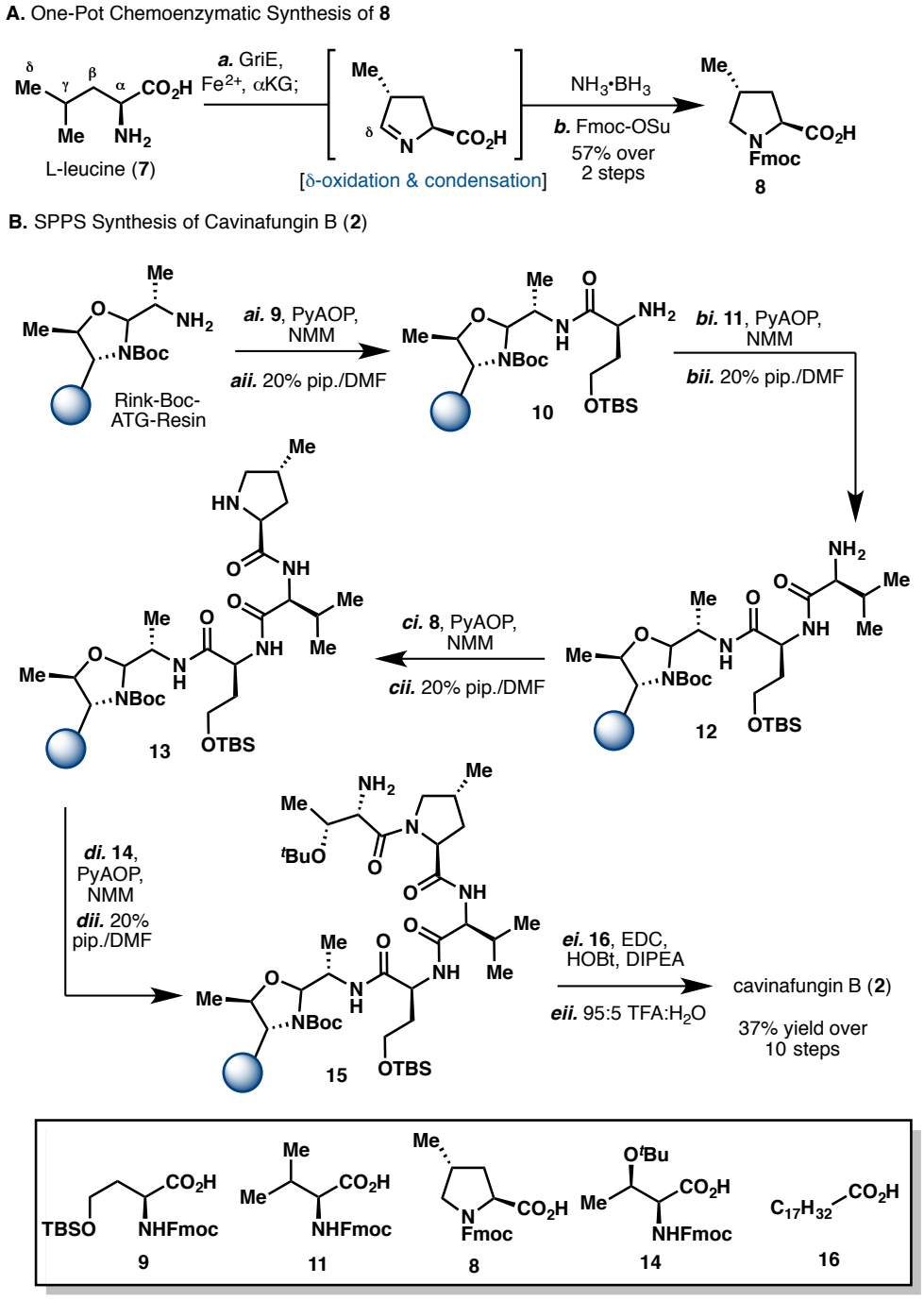


Figure 3. Chemoenzymatic synthesis of **8** and cavinafungin B.

3. Conclusions

This work describes the first synthesis of antiviral lipopetide cavinafungin B, completed in 10 steps from known intermediates. Of note, we utilized a one-pot chemoenzymatic synthesis of (2*S*,4*R*)-4-MePro to expedite the assembly of cavinafungin B. Furthermore, our strategy delineates a method for linear lipopeptide construction using Fmoc-based SPPS. With an established synthesis of cavinafungin B, this work opens the door towards further studies involving this useful family of lipopeptides. Our synthetic strategy highlights the value of incorporating chemoenzymatic steps in complex molecule synthesis.[17]

4. Experimental

4.1. General materials and methods

Unless otherwise noted, all chemicals and reagents for chemical reactions were purchased at the highest commercial quality and used without further purification. SPPS reaction vessels were purchased from Torviq. SPPS was performed using 100-200 mesh Rink Amide-Am Resin (0.55 mmol/g) purchased from Creosalus (SA6061). A KJ-201BD orbital shaker or New Brunswick™ Innova® 42/42R incubator shaker was used for the general mixing and agitation of solid-phase reactions. Reactions were monitored by thin layer chromatography (TLC) and liquid chromatography/mass spectrometry (LC/MS). TLC was performed with 0.25 mm E. Merck silica plates (60F-254) using short-wave UV light as the visualizing agent, and ninhydrin, $KMnO_4$, or phosphomolybdic acid and heat as developing agents. LC/MS was performed with Agilent 1260 Infinity System equipped with Poroshell 120 EC-C18 column (3.0 x 50 mm, 2.7 micron). Flash column chromatography was performed using an Biotage® Isolera One automated purification system loaded with Zip KP-Sil cartridges filled with SilicaFlash® P60 silica gel (230-400 mesh). Preparative HPLC was performed on Shimadzu LC-8A system equipped with SunFire C18 OBD column (30 x 250 mm, 10 micron). NMR spectra were recorded on a Bruker spetrometer and calibrated using residual undeuterated solvent. Optical rotations were measured on Autopol IV polarimeter (Rudolph Research Analytical). Enzymes (DpnI, Q5 polymerase) were purchased from New England Biolabs (NEB, Ipswich, MA). Expression and purification of GriE were performed by following previously reported protocols.[11a]

*4.2. Synthesis of N-(((9H-fluoren-9-yl)methoxy)carbonyl)-O-(tert-butyldimethylsilyl)-L-homoserine (**9**)*

Fmoc-L-homoserine (1.0 g, 2.93 mmol, 1.0 eq) and imidazole (399 mg, 5.86 mmol, 2.0 eq) were dissolved in anhydrous DMF (3 mL, 1.0M). *tert*-Butyldimethylsilyl chloride (883 mg, 5.86 mmol, 2.0 eq) was then added portionwise at 23 °C. The reaction was stirred for 9 hours, then diluted with H_2O (~20 mL) and EtOAc (~20 mL). The aqueous layer was separated and extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with sat. aq. NaCl (30 mL), dried over $MgSO_4$, and concentrated *in vacuo*. Purification by silica gel chromatography (1:9 EtOAc:hexanes to EtOAc via gradient elution) afforded TBS ether **9** (0.609 g, 46% yield) as a slightly tan foam. ^{1}H NMR spectra of **9** matches those reported in the literature.[18]

4.3. Synthesis of (2*S*,4*R*)-1-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-4-methylpyrrolidine-2-carboxylic acid *(**8**)*

A 100-mL beaker was charged with L-leucine (105 mg, 0.80 mmol, 1.0 equiv, 20 mM final concentration), L-ascorbic acid (70 mg, 0.40 mmol, 0.5 equiv), α-ketoglutaric acid (disodium salt dihydrate, 542 mg, 2.40 mmol, 3.0 equiv), followed by 40 mL of 50 mM kPi buffer (pH 7.0). After addition of 200 µL of 200 mM $FeSO_4$ solution (0.040 mmol, 0.05 equiv), the reaction was started by the addition of GriE stock solution (final concentration = 0.030 mM, 0.0015 equiv). The mixture was shaken at 20 ºC, 150 rpm. After 2 h, solid NH_3•BH_3 (61.5 mg, 2.0 mmol, 2.5 equiv) was added and the reaction was shaken for 4 h. The reaction was acidifed with 1 M HCl (1.0 mL), and centrifuged at 15,000 rpm for 5 min. The supernatant was collected, lyophilized, and used for the next step without further purification.

The crude material from the previous step was suspended in 8.0 mL of sat. aq. $NaHCO_3$. A solution of Fmoc-OSu (675 mg, 2.0 mmol, 2.5 equiv) in MeCN (4 mL) was added, and the reaction was stirred at rt overnight. The reaction was acidified with 1 M HCl until pH = 2–3. The aqueous layer was extracted with EtOAc (3 x 10 mL), and the combined organic layers were washed with sat. aq. NaCl, dried over $MgSO_4$, and concentrated *in vacuo*. Purification by silica gel chromatography (1:2 EtOAc:hexanes to 1:0.05 EtOAc:AcOH via gradient elution) afforded **8** as a white foam (161 mg, 57% yield over 2 steps). ^{1}H NMR spectra of **8** match those reported in literature.[20]

*4.4. Synthesis of cavinafungin B (**2**)*

Rink-Boc-ATG-Resin was prepared as previously reported and stored at 0 °C after lyophilization.[12b,21] Rink-Boc-ATG-Resin (100 mg, 0.0282 mmol, substitution = 0.282 mmol/g, 1.0 eq) was placed in a 2-mL reaction vessel and swollen with anhydrous DMF (3 mL) for 0.5 h. A solution of *O*-TBS-Fmoc-L-homoserine (**9**) (51 mg, 0.113 mmol, 4.0 eq), PyAOP (59 mg, 0.1128 mmol, 4.0 eq), and *N*-methylmorpholine (NMM) (0.025 mL, 0.226 mmol, 8.0 eq) in anhydrous DMF (0.30 mL, ~ 0.1M) was pre-mixed for 10 min in a scintillation vial. The reaction vessel was purged of DMF, filled with the pre-mixed solution, and agitated on an orbital shaker at 23 °C for 5 hours. The resin was washed with DMF (3 x 3 mL), DCM (3 x 3 mL), and DMF (3 x 3 mL) and capped with a solution of acetic anhydride/pyridine (1 mL, 1:9 v/v) for 10 min. The resin was washed with DMF (3 x 3 mL), DCM (3 x 3 mL), and DMF (3 x 3 mL), then treated with 20% piperidine/DMF (3 mL, 2 x 10 min) to effect Fmoc deprotection and washed with DMF (3 x 3 mL), DCM (3 x 3 mL), and DMF (3 x 3 mL). This procedure was repeated for three subsequent amide couplings and Fmoc-deprotections with Fmoc-L-valine (**10**) (38 mg, 0.113 mmol, 4.0 eq – agitated 24 h), Fmoc-(*2S*,*4R*)-Me-L-Pro (**8**) (40 mg, 0.113 mmol, 4.0 eq – agitated 24 h), and *O*-^{t}Bu-Fmoc-L-threonine (**11**) (45 mg, 0.113 mmol, 4.0 eq – agitated 24 h). A solution of oleic acid (**12**) (0.037 mL, 0.116 mmol, 3.9 eq), EDC (27 mg, 0.141 mmol, 5.0 eq), HOBt (22 mg, 0.141 mmol, 5.0 eq), and DIPEA (0.025 mL, 0.141 mmol, 5.0 eq) in anhydrous DMF (0.30 mL, 0.1 M) was pre-mixed for 10 min in a scintillation vial. The reaction vessel was filled with the pre-mixed solution, and agitated on an orbital shaker at 23 °C for 24 h. The resin was washed with DMF (3 x 3 mL), DCM (3 x 3 mL), and DMF (3 x 3 mL), and capped with a solution of acetic anhydride/pyridine (1 mL, 1:9 v/v) for 10 min. The resin was washed with DMF (3 x 3 mL), DCM (3 x 3 mL), and DMF (3 x 3 mL). A mixture of TFA/H_2O (95:5 v/v) was added to the resin and agitated for 2 h (take caution, as the solution experiences a strong exotherm). The cleavage solution was collected in a scintillation vial and the resin washed with TFA (3 x 2 mL) and DCM (3 x 2 mL). The resulting solution was dried under stream of air. The resulting film was dissolved in H_2O:MeCN (~1:1 v/v, 4 mL) and purified by HPLC (5–100% H_2O:MeCN for 35 min then 100% MeCN for 5 min, flow rate = 50 mL/min) yielding cavinafungin B (2) (7.7 mg, 37% yield) as a white powder (t_R = 32.1 min). NMR spectra of **2** match those reported in literature.[1] $[\alpha]_D$ = – 39.0 (*c* 0.4, $CHCl_3$); ^{1}H NMR (700 MHz, DMSO-d_6)

δ 9.35 (dd, *J* = 3.5, 0.7 Hz, 1H), 8.26 (dd, *J* = 19.9, 6.6 Hz, 1H), 7.90 – 7.78 (m, 2H), 5.32 (m, 2H), 4.49 – 4.44 (m, 1H), 4.41 (m, 1H), 4.38 – 4.27 (m, 1H), 4.12 – 4.03 (m, 2H), 3.81 (m, 2H), 3.41 (m, 1H), 3.26 (m, 1H), 2.00 – 1.95 (m, 4H), 1.82 (m, 1H), 1.71 (m, 1H), 1.64 (bs, 1H), 1.45 (bs, 2H), 1.30 – 1.2 (m, 20H), 1.15 (dd, *J* = 7.2, 5.2 Hz, 1H), 1.08 (d, *J* = 6.3 Hz, 1H), 0.98 – 0.94 (m, 3H), 0.89 – 0.79 (m, 9H); ^{13}C NMR (176 MHz, DMSO-d_6) δ 201.0, 172.2, 171.9, 171.5, 170.8, 169.5, 129.6, 129.6, 66.9, 59.3, 59.2, 57.9, 57.5, 54.0, 53.8, 36.5, 34.8, 34.8, 32.0, 31.3, 29.1, 29.1, 29.0, 28.8, 28.7, 28.7, 28.6, 28.6, 26.6, 25.2, 22.1, 19.3, 19.1, 18.0, 17.1, 13.5; HRMS (ESI): calc for $C_{40}H_{72}N_5O_8$: 750.5381; found 750.5146.

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