JOURNAL OF NATURAL PRODUCTS

ARTICLE

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Isolation, Structure, and Biological Activities of Fellutamides C and D from an Undescribed *Metulocladosporiella* (Chaetothyriales) Using the Genome-Wide *Candida albicans* Fitness Test

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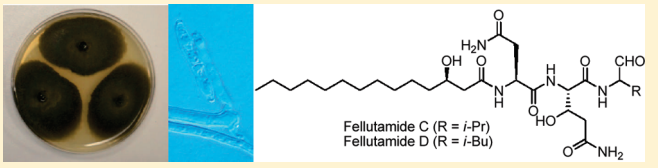
Supporting Information

ABSTRACT: In a whole-cell mechanism of action (MOA)-based screening strategy for discovery of antifungal agents, *Candida albicans* was used, followed by testing of active extracts in the *C. albicans* fitness test (CaFT), which provides insight into the mechanism of action. A fermentation extract of an undescribed species of *Metulocladosporiella* that inhibited proteasome activity in a *C. albicans* fitness test was identified. The chemical genomic profile of the extract contained hypersensitivity of heterozygous deletion strains (strains that had one of the genes of the diploid genes knocked down) of genes represented by multiple subunits of the 25S proteasome. Two structurally related peptide aldehydes, named fellutamides C and D, were isolated from the extract. Fellutamides were active against *C. albicans* and *Aspergillus fumigatus* with MICs ranging from 4 to 16 μg/mL and against fungal proteasome (IC_{50} 0.2 μg/mL). Both compounds showed proteasome activity against human tumor cell lines, potently inhibiting the growth of PC-3 prostate carcinoma cells, but not A549 lung carcinoma cells. In PC-3 cells compound treatment produced a G2M cell cycle block and induced apoptosis. Preliminary SAR studies indicated that the aldehyde group is critical for the antifungal activity and that the two hydroxy groups are quantitatively important for potency.



Natural products continue to provide a rich chemical diversity of biologically active molecules. The conventional strategy of natural product discovery has been seriously challenged by the frequent rediscovery of known molecules.[1] One of the keys to enhance success in natural products research is to identify mechanistic imprints present in the crude extract prior to chemical fractionation so that the chemical resources will be allocated to those extracts with potential novel biological activities and hence likely to yield novel chemical structures. We have implemented a chemical genomic approach using the principal human fungal pathogen *Candida albicans* that genetically profiles mechanistic imprints of biologically active molecules from various sources. As part of our effort to discover new antimicrobial compounds, we also expanded our isolation of microorganisms from diverse geographical regions and habitats and improved high-throughput fermentation methods by miniaturization and automation.[2]

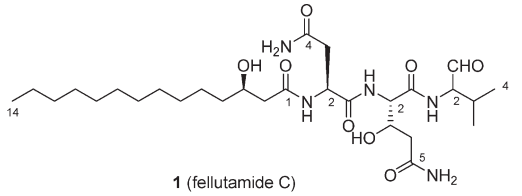
C. albicans and other *Candida* spp. are the most significant causes of fungal infections in the hospital setting worldwide in terms of total number of cases and mortality.[3] The standard antifungal therapy is restricted to drugs in three mechanistic categories, namely, azoles (inhibiting ergosterol biosynthesis), macrocyclic polyenes (perturbing the cell membrane), and cyclic lipopeptide echinocandins (inhibiting β-1,3-glucan synthesis). All treatment options have limitations including resistance, toxicity leading to a very narrow therapeutic window, and/or lack of oral efficacy, thus creating a serious need for new antifungal agents.

Our chemical genomic approach, the *C. albicans* fitness test (CaFT, adopted from an analogous assay developed in the yeast *Saccharomyces cerevisiae*),[4] relies on the nature of obligatory diploidy of the fungus. For a vast majority of genes of the pathogens, heterozygous deletion of the genes results in no detectable growth defects of the pathogens under standard conditions. However, in the presence of sublethal concentrations of an antifungal compound, only some heterozygotes display growth/fitness variations (hypersensitivity or resistance) with significant deviation from the rest of the heterozygotes. In many cases, they include those corresponding to the specific target, and in the other cases they represent aspects of the mechanism of action (MOA) of the compound.[5−10] The strains with significant deviations in their growth induced by the chemical perturbation collectively comprise the chemical genetic profile of a given antifungal compound.

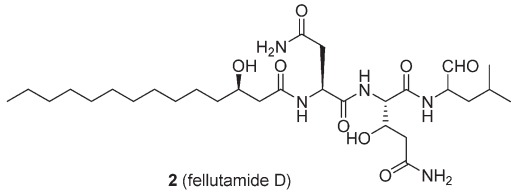
Received: February 16, 2011
Published: July 13, 2011

As the assay contained ~2900 strains in the published version[5] and ~5400 (i.e., ~90% genomic coverage for essential genes) in the upgraded version, it is possible to genetically profile an antifungal compound of interest at the genomic level. Applying this approach to crude extracts of natural products, we were able to discern novel biological activities present in the crude extracts, which in turn led to the discovery of novel chemical structures.[7,11,12] For example, we identified an extract with a chemical genomic profile indicative of the RNA cleavage/polyadenylation complex as the target. Although the profile of the extract overlapped with that of cordycepin (an analogue of adenosine), the latter contained specific resistance of a heterozygous deletion strain for the fungal nucleoside transporter, an indication of uptake of cordycepin by this transporter, and thus differentiated itself. The purified compound from this extract is a novel isoxazolidinone that inhibits the RNA poly(A) polymerase.[7,11]

Applying the same approach we identified a fungal fermentation extract that contained proteasome-inhibiting activity. Here we present bioassay-guided isolation and structure elucidation of two new antifungal agents, fellutamides C (**1**) and D (**2**), from the extract. Both compounds are potent inhibitors of fungal proteasome activity with broad spectrum antifungal activity. In addition, they showed significant potency against the human tumor cell line PC-3.



1 (fellutamide C)



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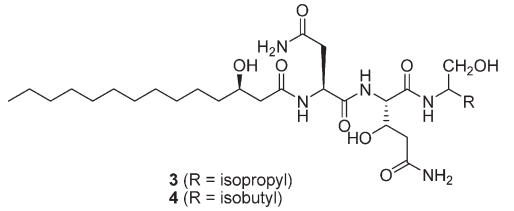

3 (R = isopropyl)
4 (R = isobutyl)

RESULTS AND DISCUSSION

CaFT Profiling of the Fungal Extract ECC2567. In an attempt to discover novel antifungal agents from microbial sources, we screened and tested extracts of fermentation broths exhibiting whole-cell *Candida* activity (see below) in the *C. albicans* fitness test. The extract under investigation, ECC2567, generated a profile indicating the target as the fungal 26S proteasome. It induced specific hypersensitivity of heterozygotes for genes of several subunits (see below for details) (Figure 1A). The in vitro proteasome assay confirmed the potent inhibitory effect of ECC2567, with MIC ≤ 0.25% WBE (whole-broth equivalents, Figure 1B). Gliotoxin is known to inhibit the chymotrypsin-like activity of the 20S proteasome in vitro[13−15] and inhibited the fungal proteasome

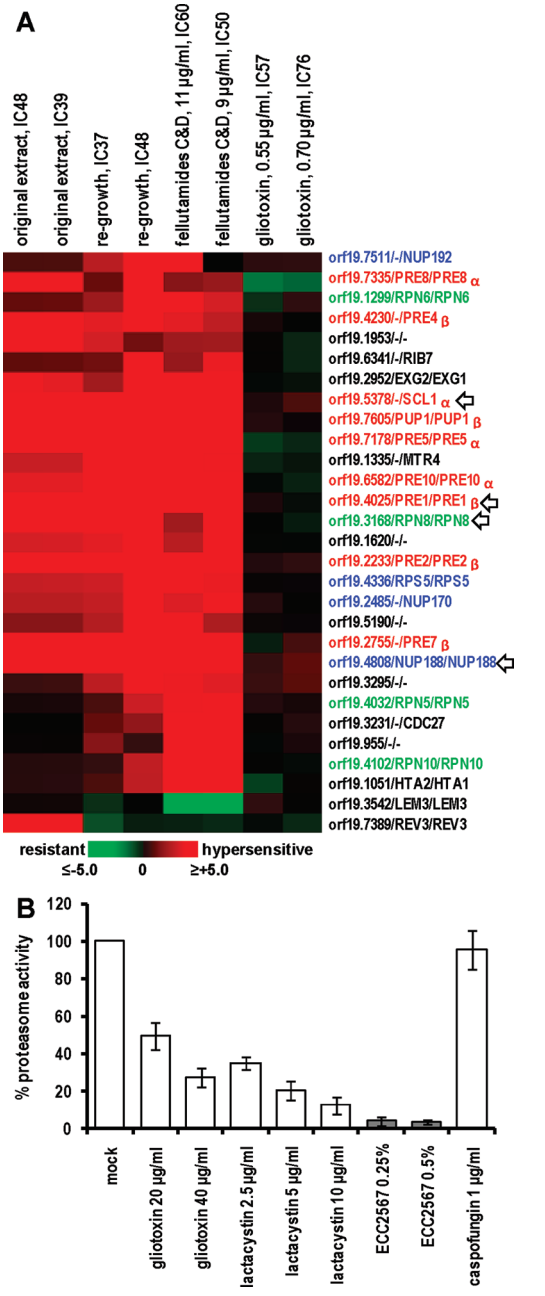

Figure 1. Chemical genomic profiling and characterization of extract ECC2567. (A) CaFT profiles of the original extract (ECC2567), regrowth extract, mixture of fellutamides C and D, and gliotoxin. Two independent CaFT experiments were selected for each extract/compound, with IC indicated on the top. In the hierarchical clustering analysis, heterozygous deletion strains were selected on the basis of the absolute values of their normalized z-scores ≥4.0 in at least two experiments. The result is displayed by heat map with scale indicated at the bottom. The genes for subunits of the 20S core particle are indicated in red (with α, β indicating the rings), the 19S regulatory particle in green, and those involved in nuclear transport in blue. The arrows indicate the genes whose heterozygous deletion strains were tested in Figure 3A and B. (B) In vitro assay for the fungal proteasome activity. The activities in the presence of compounds at the concentrations indicated were normalized with that of mock treatment. Shown are averages of three measurements with bars indicating standard deviations. Note that the antifungal MIC of gliotoxin was ~4 μg/mL, and lactacystin was not active against *C. albicans* (at 200 μg/mL).

dx.doi.org/10.1021/np2001573 |*J. Nat. Prod.* 2011, 74, 1721–1730

activity at concentrations much higher than the whole-cell activity (Figure 1B). It generated a nonoverlapping profile in the fitness test (Figure 1A). These results indicated that the antifungal activity of this extract targets the proteasome and is distinct from that of Gliotoxin and lactacystin (another proteasome inhibitor but produced by *Streptomyces* sp.), thus prompting us to isolate the active component(s) present in ECC2567.

Producing Fungus and Fermentation. The producing organism of extract ECC2567, F-192,783 (Figure S1, Supporting Information), was isolated from a soil sample collected from Equatorial Guinea. Sequence analysis of the rDNA (28s D1-D2 and ITS regions, Figures S2, S3, Supporting Information) indicated that it was closely related to and possibly congeneric with a complex of species in the genus *Metulocladosporiella* (Ascomycotina, Chaetothyriales), including *M. musae*, *M. musicola*, and the closely related *Cladosporium adianticola*.[16] In vitro, the fungus produces melanized micronematous to macronematous conidiophores (up to 150 μm tall) that terminate apically in two to three levels of polyblastic metulae that give rise to acropetal chains of conidia. The conidia are fusoid to cylindrical, smooth, hyaline to only faintly melanized, predominantly 1-sepate, mostly 15−50 μm long by 5−10 μm wide, with the extremes narrowing to an apical scar (Figure S1, Supporting Information). The consistently septate conidia easily distinguish the fungus from either *M. musae* or *M. musicola*, and in some aspects, the fungus more closely resembles *C. adianticola*, a fungus known only from foliage of an *Adiantum* sp. (Pteridaceae) from Cuba.[17] However, the conidiophores of *C. adianticola* branch in a more open pattern, and its septate conidia are more ellipsoidal, ovoid, or subglobose. Alignments and bootstrapped neighbor-joining analysis of the both the ITS and D1-D2 regions of the rDNA of this new fungus (Figures 2, 3 and Supporting Information) support the conclusion that it is distinct.

The antifungal activity produced by F-192,873 was initially detected in a pilot study that compared microfermentation in the 96-deepwell plates[2] with fermentations in the standard 12 mL

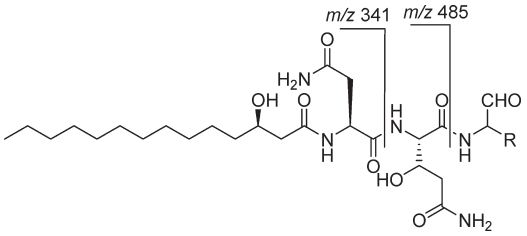

Figure 2. ESIMS fragmentation of fellutamides C (**1**) (R = isopropyl) and D (**2**) (R = isobutyl).

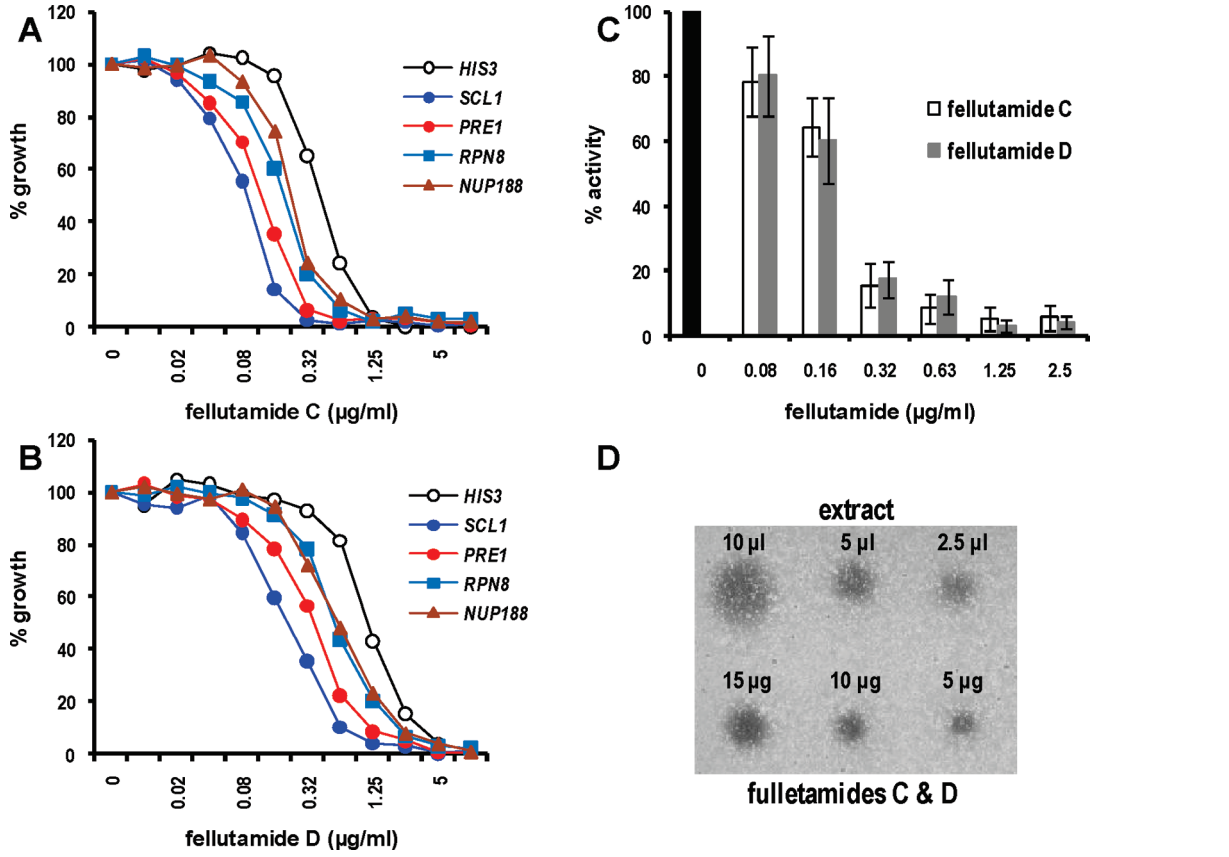

Figure 3. Characterization of fellutamides C (**1**) and D (**2**). (A and B) Selected heterozygous deletion strains (see Figure 1A) and the control (HIS3) were tested against fellutamides C and D at the concentrations indicated in YNBD. The growth of each strain in the presence of fellutamide was normalized by that of mock treatment. Shown are averages of three measurements with coefficient variation less than 10% in most cases (not shown). (C) In vitro fungal proteasome assay. The proteasome activity in the presence of fellutamide was normalized by that of mock treatment. Shown are averages of three measurements with bars indicating standard deviations. (D) Anti-aspergillus activity of fellutamides C and D (in mixture). *A. fumigatus* strain CEA10 was seeded in solid ACM medium with aliquots of compound/extract (ECC2567) with indicated amounts spotted on the surface. Shown is 24 h growth at 37 °C.

dx.doi.org/10.1021/np2001573 |*J. Nat. Prod.* 2011, 74, 1721–1730

vials.[18] The study employed 320 newly isolated fungal strains grown in three and 12 media in aerated vial fermentations and in static 96-deepwell microfermentations, respectively. Consequently, 52 of the 320 strains (16%) produced antifungal activities in deepwell microfermentations, compared to 24 (7.5%) in vial fermentations. The antifungal activity of F-192,873 was detected in only one medium among 15 different vial and deepwell microfermentations. The production of antifungal activity by F-192,873 was extremely sensitive to the growth conditions, and activity was produced only in one medium (YES) in a static microfermentation. The fermentation was scaled-up by growing the fungus in 200 mL of static liquid YES in 2.8 L Fernbach flasks for 14 days. Aerated fermentations in the same medium and under otherwise the same conditions were inactive.

Isolation of Fellutamides C and D. The fermentation broth was extracted with methyl ethyl ketone (MEK). When tested in the CaFT, the extract reproduced the original profile (Figure 1A), suggesting the preservation of the same (or similar) biological activities in the MEK extract, which was then processed by liquid−liquid extraction with EtOAc and water. The EtOAc extract, which retained the antifungal activity, was chromatographed on Sephadex LH20. The resulting antifungal fractions were further separated on an Amberchrome column, followed by reverse-phase HPLC to yield fellutamides C (10.4 mg, 4.2 mg/L) and D (7.5 mg, 3.1 mg/L). Both compounds showed poor solubility in most of the solvents tested, particularly in aqueous systems, leading to low recovery from HPLC. Prior to HPLC, a mixture of both compounds was tested in the CaFT and generated a profile that is largely consistent with those of the original and regrowth extracts (Figure 1A), suggesting that they were the components in the extracts associated with the specific biological activities sought.

Structural Elucidation of Fellutamides C and D. HRESIFTMS analysis of fellutamide C (**1**) showed a molecular formula of $C_{28}H_{51}N_5O_8$. Its UV spectrum exhibited end absorption, and the IR spectrum showed absorption bands for carbonyl and hydroxy groups. The ^{13}C NMR spectrum (Table 1) showed five carbonyl carbons in the amide carbonyl region along with three resonances appearing in the region of α-carbons typical of amino acids, suggesting that this compound was a peptide. Analysis of the DEPT spectrum revealed the presence of three methyls, six methines, 13 methylenes, and an aldehyde (Table 1). The ^1H NMR spectrum in a mixture of DMSO-d_6 + CD_3CO_2D suggested a large methylene envelope indicating the presence of a fatty chain along with the resonances for three doublets for amide NH protons and four amide NH singlets assignable to two primary amides. The COSY spectrum of **1** showed the spin systems assignable to a valinal, a β-hydroxyglutamine (HyGln), asparagine (Asn), and a β-hydroxytetradecanoic acid (HTDA, hydroxy myristic acid) (Table 1). The HRESIFTMS spectrum showed fragment ions corresponding to the loss of a molecule of water and sequential losses of valinal (m/z 485) and HyGln (m/z 341), indicating that the valinal was present at the carboxy end and the Asn formed the N-terminus, which was acylated with HTDA (Figure 2). The sequence was confirmed by the HMBC correlations of the α-CH and NH to the corresponding carbonyl carbons (Table 1).

HRESIFTMS analysis of fellutamide D (**2**) showed a molecular formula of $C_{29}H_{53}N_5O_8$ consisting of an additional methylene moiety compared to fellutamide C (**1**). The NMR spectra of **2** were identical to the corresponding spectra of **1** except for the presence of an extra methylene, which was assigned to the β-carbon of leucinal, as determined by the COSY spectrum. The mass spectrum showed a fragmentation pattern similar to **1** except for changes attributable to the substitution of valinal with leucinal in **2** (Figure 2). Epimerization of C-2 of valinal and leucinal was noticed for compounds **1** and **2** during purification.

The absolute configuration of the amino acids and the HTDA was deduced as follows. A mixture of the two compounds (**1** and **2**) was reduced to that of a mixture of corresponding primary alcohols (**3** and **4**) by reaction with $NaBH_4$. The mixture of the alcohols was hydrolyzed by 6 N HCl to yield components, leucinol, valinol, aspartic acid, 3-hydroxyglutamic acid, and 3-hydroxy myristic acid. 3-Hydroxymyristic acid was recovered by extraction with $CHCl_3$. The resulting mixture of amino alcohols and amino acids was reacted with Marfey's reagent and compared by HPLC with the Marfey's derivatives of both enantiomers of corresponding amino acids/alcohols. This led to the identification of L-valinol, L-leucinol, L-aspartic acid (L-asparagine), and 3*R*-L-glutamic acid (3*R*-L-glutamine). The recovered 3*R*-hydroxymyristic acid was purified by RP HPLC and characterized by NMR and MS. The absolute configuration was confirmed by comparison of specific rotation (observed $[\alpha]_D$ −10, lit. $[\alpha]_D$ −16). Thus the structures **1** and **2** were assigned to fellutamides C and D, respectively.

These compounds are very closely related to fellutamides A and B isolated from *Penicillium fellutanum* recovered from the gastrointestinal tract of the fish *Apogon endekataenia*.[19] Both fellutamides A and B contain (3*R*)-hydroxydodecanoic acid and leucinal. Fellutamide A contains a glutamine, and fellutamide B contains a hydroxyglutamine. In addition the other structural differences between fellutamides B and D (**2**) lie in the length of β-hydroxyl aliphatic tails (C_{14} in B and C_{12} in D). Fellutamide B is known to inhibit human proteasome and induce nerve growth factor synthesis[20] and is a potent inhibitor of the *Mycobacterium tuberculosis* proteasome.[21] However, no antifungal activity has been reported for fellutamide A or B.

Synthesis of Myristyl-(*S*)-Asn-(*S*)-Gln-(*S*)-Leucinal. Fellutamides are peptide aldehydes with β-hydroxyl aliphatic tails of varying length: (3*R*)-hydroxydodecanoic acid in fellutamides A and B and (3*R*)-hydroxymyristic acid in C and D. Both fellutamides C and D contain a hydroxy-(*S*)-glutamine. We report here the total synthesis of a simpler version of the fellutamide D (Scheme 1), which provided structure−activity relationship information. The standard PyBop coupling of L-CBZ-Asn and L-Gln-O-*t*-Bu gave an excellent yield of the dipeptide **5** (Scheme 1). Deprotection of the CBZ group by hydrogenolysis afforded the dipeptide **6**, which was coupled with myristic acid to furnish myristylated dipeptide **7** in 93% yield. Deprotection of the *tert*-butyl ester with TFA followed by PyBop coupling with L-leucinol produced the tripeptide **8** in 76% yield. The primary alcohol was oxidized with SO_3−Py complex to afford the aldehyde **8** in 60% yield. The overall reaction yield (36%) of the aldehyde was excellent, but product was lost during HPLC purification due to poor aqueous solubility.

Biological Activities of Fellutamides C and D. When the mixture of fellutamides C and D was tested in the CaFT, the resulting profile reproduced the hypersensitivity of heterozygous deletion strains for genes of four α and four β subunits of the 20S proteasome core particle and four other proteins involved in nuclear transport that was observed in the original and regrowth extracts tested at comparable inhibitory concentrations (Figure 1A). In addition, the hypersensitivity of heterozygous deletion of subunits of the 19S proteasome regulatory particle extended from

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Assignments of Fellutamides C (1) and D (2) in DMSO-d_6 + CD_3CO_2D (9:1)

		1			2[a]	
	position	δ_C	δ_H, mult (J in Hz)	HMBC (H→C)	δ_C	δ_H, mult (J in Hz)
leucinal/valinal	1	201.6	9.38, d (1.5)	C-2	201.9	9.34 s
	2	63.2	3.94, ddd (7.0, 5.5, 1.5)	C-1, 3, 4, 5, HyGln (C-1)	56.8	4.01, m
	3	27.9	2.15, m		36.4	1.47, m
	4	18.1	0.88, d (7.0)	C-2, 3, 5	23.8	1.65 m
	5	19.0	0.84, d (7.0)	C-2, 4, 4	21.4	0.85, d (7.0)
	6				23.1	0.85, d (7.0)
	NH		8.07, d (7.0)	HyGln (C-1)		8.21, d (7.0)
β-hydroxyglutamine (HyGln)	1	170.3			170.4	
	2	57.0	4.27 dd (8.5, 3.0)	C-1, 3	57.0	4.23, dd (8.5, 3.0)
	3	67.7	4.35, brm		67.5	4.37, brm
	4	39.8	2.2, m	C-3	40.0	2.20, m
	5	172.3			172.4	
	NH		7.75, d (8.5)	Asn (C-1)		7.78, d (8.5)
	OH		5.12, brs			5.14 brs
	NH_2		6.84, s	C-4		6.82, s
			7.15, s	C-5		7.15, s
asparagine (Asn)	1	171.0			171.4	
	2	49.9	4.55, apparent q (7.0)	C-1, 2, HTDA (C-1),	49.9	4.5, apparent q (7.0)
	3	36.9	2.45, m	C-2, 4	36.9	2.50, m
			2.55, dd (15, 7.0)			2.58, dd (14, 7.0)
	4	171.9			172.0	
	NH		8.16, d (7.0)	HTDA (C-1)		8.20, d (6.5)
	NH_2		6.94, s	C-3		6.95, s
			7.45, s	C-4		7.47, s
3-hydroxy-teradecanoic acid (HTDA)	1	171.4			171.4	
	2	43.5	2.2, m	C-1, 4	43.4	2.2, m
	3	67.4	3.75, brs	C-1, 5	67.5	3.77, brs
	4	37.0	1.3, m		36.9	1.33, m
			1.2, m			1.20, m
	5	25.2	1.2, m		25.2	1.29−1.35
	6	28.8	1.2, m		28.8	1.29−1.35
	7−11	29.1	1.2, m		29.1	1.29−1.35
	12	31.4	1.2, m		31.4	1.29−1.35
	13	22.1	1.2, m		22.1	1.29−1.35
	14	14.0	0.9, t (7.5)	C-12, 13	14.0	0.85, t (7.0)

[a] Compound **2** showed HMBC data similar to **1** (data not listed).

two (*orf1299/RPN6* and *orf19.3168/RPN8*) to four (plus *orf19.4032/RPN5* and orf19.4102/*RPN10*). We concluded that these two compounds were largely responsible for the chemical genetic profile of the extract and hence the biological activities present in the original fermentation extract.

The 26S proteasome of eukaryotes, consisting of the 20S core particle and the 19S regulatory particle, is an ATP-dependent proteolytic complex responsible for ubiquitin-dependent protein degradation.[22] The core particle, in turn, is comprised of two inner β rings (prominently catalytic) and two outer α rings (largely structural), with each ring containing seven subunits. The regulatory particle consists of 19 subunits, of which 10 form the base that binds to the α ring and the remaining nine constitute the lid to which polyubiquitin is bound. Fellutamides C (**1**) and D (**2**) induced specific hypersensitivity of heterozygous deletion strains for subunits of both rings of the core particles and the four subunits of the lid of the regulatory particles (Figure 1A). Collectively they suggest that the cellular target of fellutamides C and D is the fungal proteasome.

We selected four representative heterozygous deletion strains (*orf19.5378/SCL1*, an α subunit; *orf19.4025/PRE1*, a β subunit; *orf19.3168/RPN8*, a subunit of the regulatory particle; and *orf19.4088/NUP188*, a subunit of the nuclear pore complex) from the CaFT profile, as highlighted by arrows in Figure 1A, and tested them for susceptibility to both fellutamides C and D. As expected, all these strains were hypersensitive to both compounds with minor quantitative differences (Figure 3A and B). Preliminary results indicated that when compounds were tested in combination, only additive activity was observed. Although fellutamide C (**1**) was more potent than D (**2**) in the whole-cell antifungal activity, with MIC values of 2.5 and 10 μg/mL (in minimal medium YNBD), respectively (Figure 3A and B), both fellutamides were equally potent in inhibiting the proteasome activity,

dx.doi.org/10.1021/np2001573 |J. Nat. Prod. 2011, 74, 1721–1730

Scheme 1. Synthesis of Tripeptide Aldehyde (10)

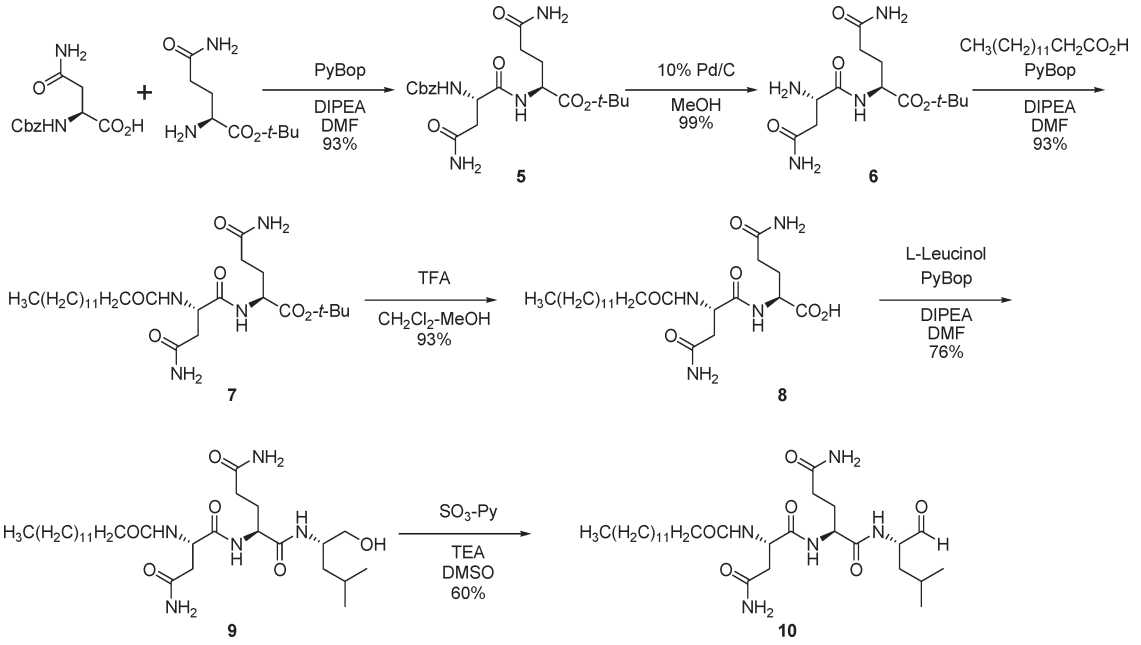

Table 2. Antifungal Activity of Fellutamides C (1) and D (2)

		MIC (μg/mL)	
organism	strain	fellutamide C	fellutamide D
C. albicans	2323	4	16
C. tropicalis	MY1012	4	8
C. glabrata	MY1381	4	8
C. lusitaniae	MY1396	16	32
C. krusei	ATCC6258	4	8
C. parapsilosis	ATCC22019	2	8
A. fumigatus	MF5668	16	32
T. mentagrophytes	MF7004	>32	>32
S. aureus	MB2865	>32	>32

with an IC_{50} of ~0.2 μg/mL (Figure 3C) and an MIC of ~1.25 μg/mL. Their proteasome activities were at least 10 times more potent than gliotoxin and lactacystin (Figure 1B). Collectively, these results suggest that the whole-cell and the proteasome inhibitory activities in the original extract are attributable to the additive effects of both fellutamides.

The antifungal spectrum of fellutamides C and D was evaluated against a panel of fungal pathogens in the medium used in the fitness test (modified RMPI[5]). Indeed, both compounds were reasonably active against all the *Candida* species tested, with MIC ranging from 2 to 32 μg/mL. Fellutamide C (**1**) was consistently more potent than D (**2**) against each fungus (Table 2). Both compounds were also active against *Aspergillus fumigatus* in liquid (Table 2) and solid (Figure 3D) media. However, no significant activity was observed against the dermatophyte *Trichophyton mentagrophytes* or the bacterium *Staphylococcus aureus* (Table 2).

We then tested the antifungal activities of the bis-des-hydroxy version of fellutamide D (i.e., compound **10**) and its leucinol version (compound **9**) using the wild-type and the heterozygous deletion strain of *PRE4/orf19.4230* (see Figure 1A), encoding another β subunit of the proteasome (Figure 4). Compound **10** showed lower activity than fellutamide D (**2**) in inhibiting the wild-type fungal cells; however both compounds showed almost equal potency against heterozygous deletion strain (*PRE4/orf19.4230* (see Figure 1A), encoding another β subunit of the proteasome (Figure 4)). Compound **9** was inactive against both strains at a concentration as high as 10 μg/mL (Figure 4). These results suggest that the β-hydroxy group of the aliphatic tail and the hydroxyl group of the central amino acid of the tripeptide are not essential for antifungal whole-cell activity and that the aldehyde group of the C-terminal amino acid is critical for biological activities.

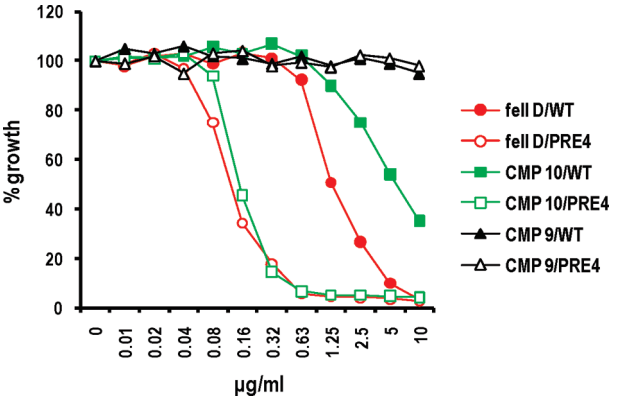

Figure 4. Antifungal activities of fellutamide D (fell D), compound **9** (CMP9), and compound **10** (CMP10) at concentrations indicated against the control (WT) and the PRE4 heterozygous deletion (PRE4) strains. The growth of each strain in the presence of drug treatments was normalized by that of the mock treatment.

The inhibition of the human proteasome by fellutamide B[20] was reported during the course of our study. As part of our examination of the properties of fellutamides C and D, we tested

dx.doi.org/10.1021/np2001573 |J. Nat. Prod. 2011, 74, 1721–1730

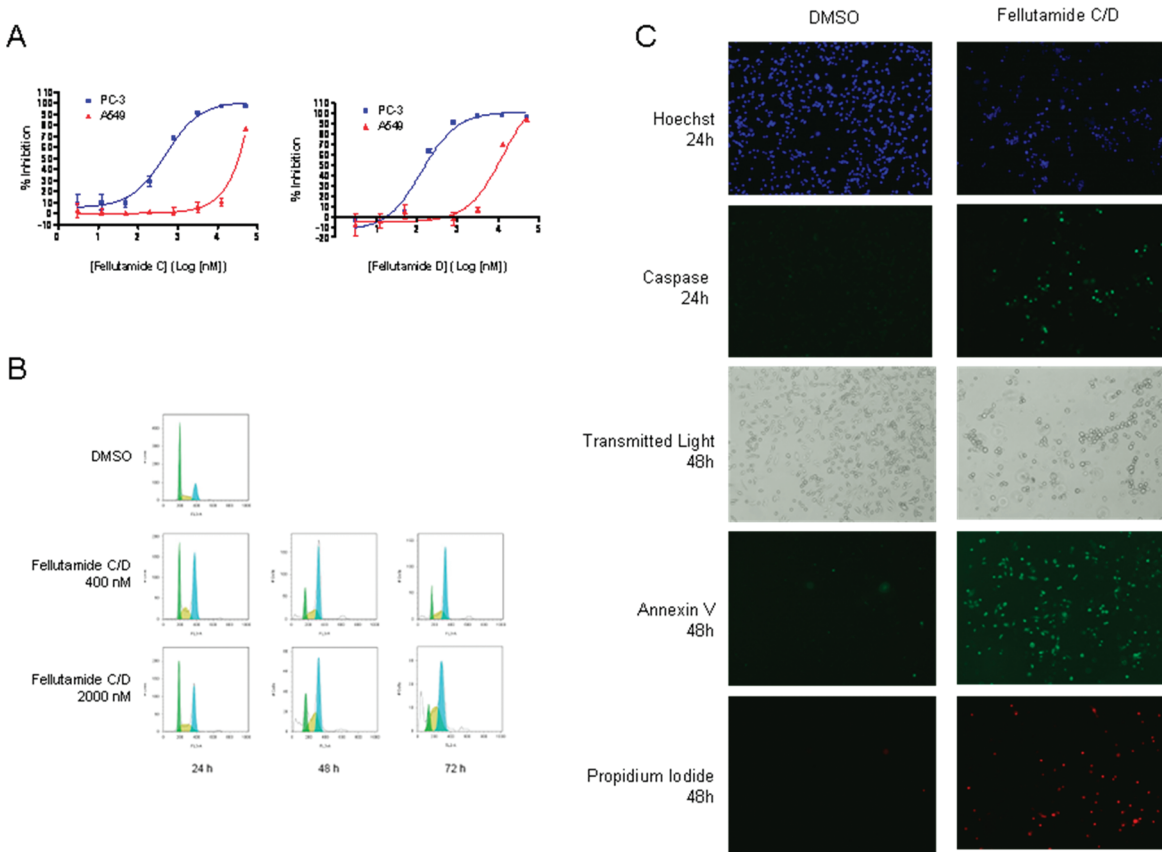

Figure 5. Effects of fellutamides C (**1**) and D (**2**) on human cancer cells. (A) Treatment with purified fellutamide C or D for 48 h results in potent inhibition of the growth of PC-3 cells (IC_{50} = 440 ± 60 and 160 ± 20 nM, respectively), while having relatively little effect on A549 cells. (B) PC-3 cells treated with fellutamide C/D are blocked at G2M, which subsequently results in apoptosis. (C) In PC-3 cells treated with fellutamide C/D/after 24 h caspase activity is apparent, and after 48 h treatment annexin V and propidium iodide staining can be seen.

the compounds for activity against PC-3 and A549 human cancer cell lines (Figure 5). PC-3 cells are known to be sensitive to proteasome inhibitors such as bortezumib, while A549 cells are relatively unaffected.[23,24] As shown in Figure 5A, both fellutamides inhibited PC-3 cells, with observed IC_{50} values of approximately 440 and 160 nM, respectively. To further characterize this effect, we then examined the cell cycle profile of PC-3 cells treated with a mixture of fellutamide C/D. In this experiment, the fellutamides produce a clear G2M block that develops within 24 h (Figure 5B). Finally, we stained fellutamide C/D-treated PC3 cells for caspase, annexin V, and propidium iodide, common markers for apoptosis and cell death (Figure 5C). Taken together, these results are consistent with proteasome inhibition within the PC-3 cells, namely, that fellutamide C/D treatment creates a G2M block and induces apoptosis.

Fellutamides are peptide aldehyde inhibitors of the proteasome and contain β-hydroxy aliphatic tails of various lengths and C-terminal aldehyde.[20] The aldehyde functional group forms a hemiacetal covalent adduct at the active site of the proteasome, leading to its inhibition.[25] However, while peptide aldehydes such as MG132 and bortezomib are potent inhibitors of the chymotrypsin-like activity of the human proteasome that results in rapid cell cycle arrest and induction of apoptosis (thus killing the cells), they do not inhibit growth of the yeast *S. cerevisiae* partially due to poor uptake.[26,27] Fellutamide B is known to inhibit the chymotrypsin-, caspase-, and trypsin-like activities of the human proteasome.[20] We confirmed that fellutamides C and D also inhibited the chymotrypsin-like activity of the human proteasome (Figure 5). As expected, both were cytotoxic against human PC-3 prostate carcinoma cells, but were not active against A549 lung carcinoma cells. In PC-3 cells, fellutamides treatment caused a G2M cell cycle arrest and induced apoptosis. This pattern of activity matches that of known proteasome inhibitors, including the tripeptide boronic acid bortezomib.[23,24,28] The human proteasome has emerged as a new therapeutic target for cancer and neurodegenerative diseases.[20,25] This raises the question whether fellutamides are suitable antifungal agents with therapeutic potential due to polypharmacy, even though their target, the fungal proteasome, is essential for virulence in an animal model of candidiasis.[29] In addition, application of fellutamides to other therapeutic areas must address the mechanistic disadvantages associated with other peptide aldehyde inhibitors. As in the case of MG132, the formation of the aforementioned covalent hemiacetal adduct between the inhibitor and the catalytic center of the proteasome is a reversible reaction under physiological conditions. The aldehyde inhibitors are rapidly inactivated by oxidation inside the cells and effluxed by the multidrug resistance carrier system.[25] If this is also true for fellutamides, their application in antifungal and other therapeutic areas is limited.

In summary, we have described the discovery of two peptide aldehyde inhibitors of fungal proteasome using the *C. albicans* fungal test from an undescribed species of *Metulocladosporiella* from Equatorial Guinea. We have demonstrated for the first time that the chemical inhibition of fungal proteasome leads to inhibition of fungal growth.

dx.doi.org/10.1021/np2001573 |*J. Nat. Prod.* 2011, 74, 1721–1730

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation was recorded with a Perkin-Elmer 241 polarimeter. UV spectra were recorded on a Perkin-Elmer Lambda 35 spectrometer. IR spectra were recorded with a Perkin-Elmer Spectrum One FT-IR spectrophotometer. All NMR spectra were recorded with a Varian Unity 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer in DMSO-d_6 or a mixture of DMSO-d_6 and CD_3CO_2D. All shifts were reported in δ (ppm) using solvent signals of DMSO-d_6 (^{1}H, 2.53; ^{13}C, 39.51 ppm) as internal standards. ^{1}H, ^{13}C, COSY, DEPT, HMQC, and HMBC spectra were measured using standard Varian pulse sequences. LRMS data were recorded on an Agilent 1100 MSD with ES ionization, and HRESIFTMS was obtained on a Thermo Finnigan LTQ-FTMS spectrometer. An Agilent HP 1100 instrument was used for analytical HPLC.

Producing Organism and Its Characterization. The producing organism (F-192,783) was isolated from a soil sample collected near Rio Campo, Equatorial Guinea, using a method for plating washed soil particles.[30] Frozen stock cultures in 10% glycerol (−80 °C) are maintained in the collection of Fundación MEDINA. Total genomic DNA was extracted from mycelia grown on YM agar. The 28S rDNA fragments, containing D1-D2[31] and ITS regions, were PCR amplified; sequence alignments[32] and phylogenetic analyses were previously described.[9] Results from morphological and phylogenetic analyses are illustrated in Supplemental Figures S1−S3.

Fermentations in Nutritional Arrays and Scale-up for Isolation. The strategy and protocols for fermentation of fungi on nutritional arrays have been described previously.[2,33] The antifungal activity, determined by the zone of inhibition of wild-type *C. albicans*, of F-192,783 in the fermentation extract of YES medium (yeast extract 20 g; sucrose 150 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01 g; $CuSO_4 \cdot 5H_2O$ 5 mg; $ZnSO_4 \cdot 7H_2O$ 10 mg; distilled H_2O 1000 mL). The scale-up fermentation employed static cultures of 200 mL of YES medium in 2.8 L Fernbach flasks at 22 °C for 14 days. The resulting fermentation broth was extracted with an equal volume of acetone, and aliquots were used in the fitness test and chemical fractionation.

***C. albicans* Fitness Test and Biological Assays.** The *C. albicans* fitness test and strains were described previously.[5] Liquid assays were performed using media indicated in the figure legends, with the starting OD_{600} of 0.02 in 96-well plates. The growth was determined by OD_{600} after 20 h growth at 30 °C and normalized by that of the same strains in the absence of drug treatment (mock). The chymotrypsin-like activity of the proteasome (Proteasome-Glo, Promega) was assessed according to the manufacturer's instruction with modifications using whole-cell extracts prepared from *C. albicans* grown in the exponential phase. Each assay contained extracts equivalent to $\sim 10^6$ cells. The antifungal MIC reported in Table 2 was measured as previously described.[9]

Human Cancer Cell Proliferation Assay. ATP Vialight (Lonza, Rockland ME) detection of viable cell mass was used to measure cell proliferation for PC-3 (ATCC #) and A549 (ATCC #) cells following the method described by Nagashima et al.,[34] with the exception that the experiments employed 8-point dose curves and final test compound concentrations of 50 000, 12 500, 3125, 781.3, 195.3, 48.8, 12.2, and 3.1 nM. IC_{50} values were calculated using a four parameter fit logistic equation of the % inhibition data plotted as a function of the log of concentration.

Flow Cytometry. Briefly, PC-3 cells were plated in 10 mm cell culture treated dishes and incubated overnight. Final concentrations of 400 or 2000 nM of fellutamide C/D (0.05% final DMSO) were added to the cells followed by incubation for 24, 48, and 72 h. After compound treatment, both floating and attached cells were collected, stained for DNA content, subjected to cell cycle analysis on a BD FACS Calibur (Becton Dickinson, San Jose, CA), and further analyzed using FlowJo7.

Flourescent Microscopy. PC-3 cells were seeded in eight-chamber glass slides and incubated overnight. Cells were treated with either 400 or 2000 nM fellutamide C/D (0.05% final DMSO) for 24 or 48 h. Following compound treatment, cells were double stained with Annexin-V (Roche Applied Science, Indianapolis, IN) and propidium iodide or with Pan Caspases (Millipore, Temecula, CA) and Hoechst nuclear dye and imaged on a fluorescent microscope.

Extraction and Purification of Fellutamides C and D. The scale-up fermentation broth (2.4 L) was extracted with 2.4 L of methyl ethyl ketone by shaking on a platform shaker for 2 h. The resulting extract was concentrated under reduced pressure to remove most of the MEK, leaving behind mostly aqueous extract (~0.7 L), which was extracted twice with EtOAc (~0.5 L each). The EtOAc extract was concentrated under reduced pressure to yield 1 g of gum. This material was dissolved in 15 mL of 1:1 MeOH/CH_2Cl_2 and charged to a 500 cm^3 Sephadex LH20 column. The column was eluted with MeOH at a flow rate of 5 mL/min. Fractions eluting from 250 to 300 mL elution volume contained all antifungal activity (of both compounds). They were pooled and concentrated to give 190 mg of residue. The dry material was dissolved in MeOH/H_2O and filtered, and the solution was charged to a 10 cm^3 Amberchrome column at 40% aqueous MeOH and eluted with a 40−100% aqueous MeOH with a 10% step gradient. All the activity was found in the 90% and 100% aqueous MeOH fractions. A small portion was tested in the fitness test. The pooled fractions together with the recovered solid from the filtration of Amberchrome charge were chromatographed by an Amberchrome Profile HPLC column (HPR10, 250 × 22 mm) using isocratic 40% aqueous CH_3CN with 0.1% TFA at a flow rate of 10 mL/min. The chromatographic loading (2−5 mg/run) of these compounds was very poor and required a number of repeated chromatographies. Pooled fractions were immediately lyophilized to give 10.4 mg of fellutamides C (4.3 mg/L) and 7.5 mg of D (3.1 mg/L).

Fellutamide C (1): $[\alpha]^{23}_D$ −10.0 (*c* 0.4, MeOH); UV(MeOH) λ_{max} end absorption; IR (ZnSe) ν_{max} 3271, 2920, 2850, 1624, 1559, 1372, 1200, 1015 cm^{-1}; ESIMS *m/z* 586 $[M + H]^+$; HRESIFTMS *m/z* 586.3807 (calcd for $C_{28}H_{51}N_5O_8$ + H, 586.3818), 568.3700 (M − H_2O), 485.30 (M − valinal), 341.24 (M − HyGln-valinal); ^{1}H and ^{13}C NMR data, see Table 1.

Fellutamide D (2): $[\alpha]^{23}_D$ −22.5 (*c* 0.4, MeOH); UV (MeOH) λ_{max} end absorption; IR (ZnSe) ν_{max} 3284, 2956, 2920, 2851, 1663, 1624, 1547, 1408, 1203, 1182, 1134, 1051, 1025 cm^{-1}; ESIMS *m/z* 600 $[M + H]^+$; HRESIFTMS *m/z* 600.3960 (calcd for $C_{29}H_{53}N_5O_8$ + H, 600.3967), 582 (M − H_2O), 485.2965 (M − leucinal), 341.2433 (M − HyGln-leucinal); ^{1}H and ^{13}C NMR data, see Table 1.

Hydrolysis and Absolute Configuration Determination. A 10.5 mg sample of the mixture of the two compounds was dissolved in 0.5 mL of MeOH, 4−5 mg of $NaBH_4$ was added at room temperature, and the mixture was stirred for 1 h. The reaction was complete, and slightly more polar products were formed. This material was diluted by addition of 1 mL of H_2O and passed through a 1 mL Amberchrome column packed in water and washed with water. The product alcohols were eluted with 100% MeOH, and the solution was concentrated to dryness, yielding 10 mg of the mixture of two peptidic alcohols. The peptidic alcohols were dissolved in 0.5 mL of 6 N HCl in a sealed tube and heated at 105 °C overnight. The solution was first diluted with 1 mL of H_2O, and 1/10 aliquot was saved for amino acid analysis using Marfey's reagent. The remaining 9/10 portion was extracted with 1 mL of $CHCl_3$, and the organic extract was concentrated to dryness, dissolved in MeOH, and purified by semipreparative HPLC (Zorbax RX C_8, 9.6 × 250 mm, 35 °C at 4 mL/min). The major peak observed in a light-scattering detector was collected and lyophilized to give 0.4 mg of powder: $[\alpha]^{23}_D$ −10 (*c* 0.2, $CHCl_3$), [(3*R*)-hydroxytetradecanoic acid lit.[35,36]], $[\alpha]_D^{25}$ −16 (*c* 1.0 $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, 7 Hz), 1.25−1.6 (16H, m), 1.43 (2H, m), 4.09 (1H, m), 2.55 (2H, m), 2.45 (2H, m).

Cbz-(*S*)-Asn-(*S*)-Gln-O-*t*-Bu (5). To a solution of CBZ-(S)-asparagine (266 mg, 1 mmol) in DMF (2 mL) were added H-glutamine-*tert*-butyl ester (202 mg, 1 mmol) and PyBop reagent (327 mg, 1.1 mmol) followed by DIPEA (561 mg, 2.2 mmol) at room temperature, and the reaction mixture was stirred. A precipitate appeared after overnight stirring, and the reaction was complete. Water (20 mL) was added, the solution was stirred for 10 min, and the product was collected by filtration through a sintered glass funnel. The product was air-dried followed by drying under vacuum to give 430 mg (yield 93%) of **5** as a colorless powder: ^{1}H NMR (DMSO-d_6) δ 8.16 (1 H, d, J = 7.4 Hz), 7.38−7.29 (6 H, m), 7.23 (1 H, brs), 6.88 (1 H, brs), 6.75 (1 H, brs), 4.99 (2 H, s), 4.40−4.34 (1 H, m), 4.06−4.01 (1 H, m), 2.46 (1 H, dd, J = 15, 4.4 Hz), 2.37 (1 H, dd, J = 15, 9.4 Hz), 2.10 (2 H, t, J = 8 Hz), 1.91−1.84 (1 H, m), 1.79−1.74 (1 H, m), 1.37 (9 H, s); ESIMS m/z 451 (M + H).

H_2N-(*S*)-Asn-(*S*)-Gln-O-*t*-Bu (6). A solution of the sparingly soluble Cbz-dipeptide **5** (230 mg) in MeOH (20 mL) was hydrogenated overnight using 10% Pd/C (10 mg) using a balloon filled with hydrogen. The catalyst was removed by filtration through a 0.45 μm filter and concentrated to give 160 mg of **6** as a colorless powder (yield 99%): ^{1}H NMR (DMSO-d_6) δ 8.12 (1H, d, J = 7.8 Hz), 7.38 (1 H, s), 7.24 (1 H, s), 6.84 (1 H, s), 6.75 (1 H, s), 4.08 (1 H, m), 3.48 (1 H, dd, J = 9.30, 3.7 Hz), 2.39 (1 H, dd, J = 15, 3.9 Hz), 2.22−2.04 (3 H, m), 1.94−1.86 (1H, m), 1.80−1.72 (1 H, m), 1.38 (9H, s); ESIMS m/z 317, (M + H).

Myristyl-(*S*)-Asn-(*S*)-Gln-O-*t*-Bu (7). To a solution of myristic acid (113 mg, 0.05 mmol) and H_2N-dipeptide **6** (142 mg, 0.45 mmol) in DMF (2 mL) was added PyBop reagent (219 mg, 0.5 mmol) followed by DIPEA (0.173 mL, 0.99 mmol). The solution was stirred at room temperrature overnight, leading to the formation of a precipitate, which was diluted with 20 mL of water and collected by filtration through a sintered funnel. The precipitate was thoroughly washed with water, dried under air followed by in vacuo to give the myristylated dipeptide **7** (230 mg, 93%) as a colorless powder: ^{1}H NMR (DMSO-d_6) δ 8.01 (1 H, d, J = 7.64 Hz), 7.91 (1 H, d, J = 8.13 Hz), 7.22 (2 H, d, J = 13.03 Hz), 6.86 (1 H, s), 6.74 (1 H, s), 4.58−4.52 (1 H, m), 4.05−3.98 (1 H, m), 2.45 (1 H, dd, J = 15, 4.5 Hz), 2.34 (1 H, dd, J = 16.12, 8.92 Hz), 2.06 (4 H, m), 1.92−1.82 (1 H, m), 1.73 (1 H, m), 1.44 (2 H, s), 1.37 (9 H, s), 1.22 (20 H, s), 0.83 (3 H, t, J = 6.9 Hz); ESIMS m/z 527 (M + H).

Myristyl-(*S*)-Asn-(*S*)-Gln-OH (8). To a solurion of myristyl-dipeptide-*tert*-butyl ester **7** (130 mg, 0.247 mmol) in 5 mL of CH_2Cl_2 and 2 mL of MeOH was added TFA (0.95 mL), and the solution was stirred overnight at room temperature. After completion of the reaction, volatile materials were removed under a stream of N_2 and the residue was dried under vacuum to give 112 mg (93%) of **8** as a yellowish powder: ^{1}H NMR (DMSO-d_6) δ 7.97 (1 H, d, J = 7.7 Hz), 7.92 (1 H, d, J = 8 Hz), 7.24 (1 H, s), 7.19 (1 H, s), 6.85 (1 H, s), 6.73 (1 H, s), 4.54 (1 H, td, J = 8, 5 Hz), 4.12 (1 H, td, J = 8.2, 5 Hz), 2.42 (1 H, dd, J = 15, 4.5 Hz), 2.33 (1 H, dd, J = 15.5, 8.4 Hz), 2.18−2.02 (4 H, m), 1.96−1.87 (1 H, m), 1.79−1.69 (1 H, m), 1.44 (2 H, s), 1.22 (20 H, s), 0.84 (3 H, t, J = 6.8 Hz); ESIMS m/z 471 (M + H).

Myristyl-(*S*)-Asn-(*S*)-Gln-(*S*)-Leucinol (9). To a solution of myristylated dipeptide **8** (14.1 mg, 0.03 mmol) and (*S*)-leucinol (3.86 mg, 0.033 mmol) in DMF (1 mL) was added PyBop reagent (14.6 mg, 0.033 mmol) followed by DIPEA (0.011 mL, 0.060 mmol). The reaction mixture was stirred at room temperature for 4 h and diluted with water (20 mL). The precipitate was collected by filtration through a sintered glass funnel, washed with water, and dried under air and in vacuo to give 13 mg (76%) of **9** as a colorless powder: ^{1}H NMR (DMSO-d_6) δ 8.02 (1 H, d, J = 7.5 Hz), 7.97 (1 H, d, J = 8 Hz), 7.43 (1 H, d, J = 8.5 Hz), 7.37 (1 H, s), 7.17 (1 H, s), 6.91 (1 H, s), 6.71 (1 H, s), 4.52 (1 H, t, J = 5.5 Hz), 4.45 (1 H, q, J = 7 Hz), 4.08 (1 H, dt, J = 8, 5 Hz), 3.75 (1 H, m) 3.25 (1 H, m), 3.16 (1H, m), 2.50 (1H, dd, J = 15.5, 6.5 Hz), 2.37 (1 H, dd, J = 15.53, 7.1 Hz), 2.27−2.20 (1 H, m), 2.10−1.97 (4 H, m), 1.92−1.84 (1 H, m), 1.74−1.65 (1 H, m), 1.59−1.49 (1 H, m), 1.45 (2 H, s), 1.50−1.01 (22 H, m), 0.85 (3H, d, J = 6.5 Hz), 0.84 (3H, t, J = 7 Hz), 0.80 (3H, d, J = 6.5 Hz); ESIMS m/z 570 (M + H).

Myristyl-(*S*)-Asn-(*S*)-Gln-(*S*)-Leucinal (10). To a solution of leucinol peptide **9** (8 mg, 0.014 mmol) in DMSO (1 mL) was added TEA (0.01 mL, 0.07 mmol), and the solution was stirred at room temperature under N_2 for 20 min. It was cooled to 0 °C, and then SO_3−Py complex (11.2 mg, 0.070 mmol) was added. The reaction mixture was stirred at 0−5 °C for 30 min. The reaction was quenched with 100 uL of water, diluted with 1 mL of MeOH, and filtered through a 4.5 μm filter, and 50% was purified by reversed-phase HPLC (Zorbax RX C_{18} (21.2 × 250 mm), 37 min gradient of 10−95% MeOH(aq) at 12 mL/min). Fractions 35−37 (35−37 min) contained the desired product and were pooled. These pooled fractions were concentrated to give 2.4 mg (60%) of the aldehyde **10** as a colorless powder: ^{1}H NMR (DMSO-d_6) δ 9.33 (1 H, s), 8.28 (1 H, d, J = 7.32 Hz), 8.09 (1 H, d, J = 7.65 Hz), 8.02 (2 H, d, J = 7.40 Hz), 7.39 (1 H, s), 7.19 (1 H, s), 6.90 (1 H, s), 6.73 (1 H, s), 4.48 (1 H, q, J = 7 Hz), 4.17 (1 H, m), 4.03 (1 H, m), 2.62 (1 H, m), 2.50 (1 H, dd, J = 15, 6 Hz), 2.39 (1 H, dd, J = 15, 7 Hz), 2.34 (1 H, m), 2.08 (4 H, m), 1.94 (1 H, m), 1.75 (1 H, m), 1.61 (1 H, m), 1.46 (2 H, m), 1.22 (22 H, s), 0.87 (3 H, d, J = 6.5 Hz), 0.84 (3H, t, J = 6.5 Hz), 0.82 (3H, d, J = 7 Hz); ESIMS m/z 568 (M + H).

■ ASSOCIATED CONTENT

Supporting Information. Photograph of the producing strain (Figure S1) and phylogenetic tree (Figures S2 and S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

The authors thank M. Arocho, K. Calati, and K. Ferguson for the preliminary isolation and assay support, and members of the Center of Fungal Genetics for their contributions to CaFT screening of natural products.

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