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Article

Antibacterial and NF-κB Inhibitory Lumazine Peptides, Aspochalasin, γ-Butyrolactone Derivatives, and Cyclic Peptides from a Hawaiian *Aspergillus flavipes*

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ABSTRACT: Five new lumazine peptides (**1**−**5**), a new aspochalasin derivative (**6**), and a new γ-butyrolactone derivative (**7**), together with seven known compounds (**8**−**14**), were isolated from a Hawaiian fungal strain, *Aspergillus flavipes* FS888. Compound **1** is an uncommon natural product containing an isocyano group. The structures of the new compounds **1**−**7** were elucidated by NMR spectroscopy, HRESIMS, chemical derivatization, and ECD analysis. Compounds **12**−**14** showed significant antibacterial activity against *S. aureus* when in combination with disulfiram. Additionally, compounds **9** and **13** showed NF-κB inhibitory activity with IC_{50} values of 3.1 ± 1.0 and 10.3 ± 2.0 μM, respectively.

Fungi have been the sources of many marketed drugs and biologically active agents.[1] For examples, penicillin, the well-known first true antibiotic, and its analogues are produced from various species of *Penicillium* and *Aspergillus*;[2] the fungal metabolite mevastatin was isolated from *Penicillium citrinum*, based on which the statin atorvastatin (Lipitor) was synthesized, becoming the best-selling pharmaceutical in history in 2003;[3] and the famous immunosuppressant drug ciclosporin was first isolated from *Tolypocladium inflatum*, an ascomycete fungus.[4] Hawaii is known for its diverse although mainly tropical climate and different microenvironments, most likely due to the unique geographical location. Our studies over the past six years showed that Hawaiian fungi are a rich source of new secondary metabolites and biologically active compounds.[5−20] However, Hawaiian fungi are chemically and biologically underexplored.[20] *Aspergillus* is a large genus containing about 300 species.[21] The *Aspergillus* Secondary Metabolites Database (A2MDB) documents 807 unique secondary metabolites isolated from 675 *Aspergillus* species,[22] indicating that *Aspergillus* are excellent producers of secondary metabolites.[23]

In our search for new biologically active compounds from Hawaiian fungi,[5−20] we studied *Aspergillus flavipes* FS888. The strain was isolated from a soil sample collected at the south end of Hanauma Bay near the open ocean. An extract of FS888 (20 μg/mL) that did not show any activity when tested alone demonstrated inhibitory activity against *S. aureus* in the presence of disulfiram (20 μM). Disulfiram, an FDA-approved drug used to support the treatment of chronic alcoholism (up to 500 mg daily), is an aldehyde dehydrogenase (ALDH) inhibitor.[24] In recent years, it was reported that disulfiram showed a narrow spectrum of inhibitory activity against some Gram-positive bacteria.[25,26] Disulfiram alone exhibited antibacterial activity against *S. aureus* subsp. *aureus* Rosenbach (ATCC 29213),[25] hVISA Mu3 ATCC 700698,[26] CA-MRSA CA-347 (a methicillin-resistant *S. aureus* strain),[26] and HA-MRSA COL (a methicillin-resistant *S. aureus* strain)[26] with MIC values of 32, 32, 16, and 8 μg/mL (108, 108, 54, and 27 μM), respectively. Our results demonstrated that when tested against *S. aureus* subsp. *aureus* Rosenbach (ATCC 43300), the antibiotic adjuvant disulfiram by itself was not active at 23.68 μg/mL (80 μM). Wright and his colleagues showed that disulfiram can enhance the antimicrobial efficacy of FDA-approved antibiotics, indicating that disulfiram can sensitize pathogenic bacteria to other antibiotic agents.[25] Through assay-guided separation using disulfiram (20 μM) in our combination assay, we isolated 14 secondary metabolites, including seven new compounds (**1**−**7**), along with seven known compounds (**8**−**14**) (Chart 1) from *A. flavipes* FS888. Herein, we present the isolation, structural

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Chart 1

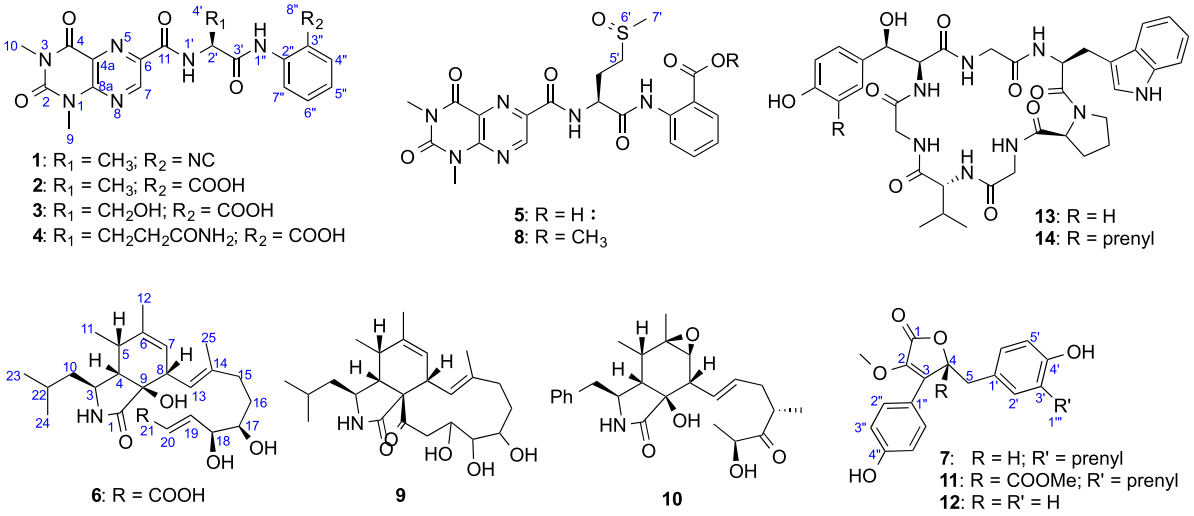

Table 1. NMR Spectroscopic Data (400 MHz) for 1−5 in DMSO-d_6

	1		2		3		4		5	
no.	δ_C	δ_H J (Hz)	δ_C	δ_H J (Hz)	δ_C	δ_H J (Hz)	δ_C	δ_H J (Hz)	δ_C	δ_H J (Hz)
2	150.4		150.5		150.4		150.4		150.4	
4	159.2		159.4		159.3		159.4		159.3	
4a	126.3		126.3		126.2		126.3		126.2	
6	139.0		139.6		139.2		139.6		139.5	
7	146.4	9.22, s	146.8	9.30, s	146.4	9.29, s	146.7	9.31, s	146.8	9.31, s
8a	149.6		149.5		149.6		149.4		149.4	
9	29.4	3.54, s	29.5	3.59, s	29.4	3.62, s	29.4	3.60, s	29.4	3.59, s
10	28.6	3.37, s	28.7	3.36, s	28.6	3.38, s	28.7	3.37, s	28.7	3.36, s
11	161.5		162.4		167.9		162.4		162.7	
1′-NH		9.14, d (7.2)		9.12, d (7.3)		8.57, d (7.9)		8.91, d (7.9)		9.12, t (6.4)
2′	48.4	4.99, m	50.2	4.68, m	56.7	4.54, m	54.3	4.56, m	53.5/53.8	4.77, m
3′	158.7		170.4		167.9		169.7		168.7	
4′	20.0	1.54 d (6.8)	17.7	1.54 d (7.2)	61.7	3.85, m 4.03, m	27.5	2.09, m 2.28, m	24.5/24.9	2.28−2.36, m 2.43−2.45, m
5′							31.5	2.16−2.18, m	49.6/49.7	2.73−2.96, m
6′							173.6			
7′									37.8/38.0	2.56, s
1″-NH						14.70, s		14.80, s		14.18
2″	148.6		140.5		140.3		140.4		140.3	
3″	121.3		119.1		124.9		125.1		123.9	
4″	125.9	8.04 d (7.9)	119.0	8.54 d (7.7)	118.5	8.51 d (8.3)	118.3	8.48 d (8.2)	118.5	8.50 d (8.1)
5″	126.0	7.42 t (7.5)	133.1	7.51 dd (7.9, 1.4)	129.9	7.27 ddd (8.5, 8.0, 1.6)	129.9	7.27 ddd (1.6, 8.0, 8.6)	130.7	7.33 t (7.6)
6″	134.0	7.74 t (7.4)	122.3	7.09 t (7.8)	121.5	6.94 t (7.5, 7.3, 1.5)	121.6	6.95 ddd (1.4, 7.6, 7.7)	121.9	6.99 t (6.7)
7″	126.7	7.58 d (8.1)	131.2	7.94 dd (7.9, 1.4)	131.5	7.93 dd (7.7, 1.6)	131.2	7.94 dd (7.7, 1.6)	131.4	8.01 d (7.9)
8″			169.4		165.3		169.6		168.7	
9″	163.3									

elucidation, plausible biosynthesis, and biological evaluation of these metabolites.

RESULTS AND DISCUSSION

Compound **1** was obtained as pale yellow oil, and its molecular formula was determined as $C_{19}H_{17}N_7O_4$ by HRESIMS, requiring 15 degrees of unsaturation. The UV spectrum showed maxima absorption at 232, 265, and 317 nm. The ^{1}H NMR spectrum of **1** showed the presence of five aromatic (or heteroaromatic) protons (5× Ar−H), three methyl groups (3× CH_3), one methine (1× CH), and two broad peaks due to two exchangeable protons (Table 1). The ^{13}C NMR and HSQC spectra of **1** demonstrated the presence of 19 carbons including three methyl (3× CH_3, including 1× C−CH_3 and 2× N−CH_3), one sp^3 methine (1× CH), five sp^2 methines (5× =CH), five sp^2 nonprotonated carbons (5× =C), four carbonyl (4× −CO−N), and one sp carbon (1× ≡C). The COSY spectrum

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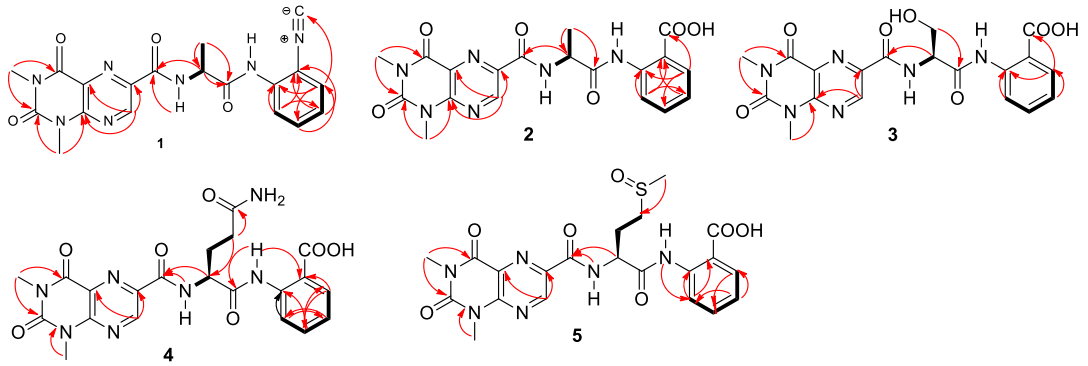

Figure 1. Key COSY (bolds) and HMBC (arrows, red) correlations of **1**−**5**.

of **1** indicated the existence of two spin systems, one (CH_3−CH) in the relatively high field and another one in the aromatic region (CH═CH−CH═CH, 1,2-disubstituted benzene) (Figure 1). In the HMBC spectrum of **1**, the methyl doublet showed correlations to the sp^3 methine (δ_C 48.4) and a carbonyl (δ_C 158.7), indicating the presence of an alanine residue. The two methyl singlets (N^1−CH_3 and N^3−CH_3) correlated to C-2 (δ_C 150.4), C-4 (δ_C 159.2), and C-8a (δ_C 149.6) (Figure 1), indicating the presence of a 5,6-disubstituted 1,3-dimethylpyrimidine-2,4-dione moiety, while the heteroaromatic singlet showed HMBC correlations to C-4a (δ_C 126.3), C-6 (δ_C 139.0), and C-8a and C-11 (δ_C 161.5), indicating the presence of a 5,6-disubstituted pyrazine-2-carboxylic acid moiety, which must be merged with the 5,6-disubstituted 1,3-dimethylpyrimidine-2,4-dione moiety to form a 1,3-dimethyl 6-carboxyl lumazine fragment. HMBC correlations from $N^{1'}$-H to C-11 and C-3′ (δ_C 158.7) indicated that the 1,3-dimethyl 6-carboxyl lumazine fragment must be connected to the alanine residue, which was very similar to that of penilumamide D.[27] The chemical composition of the 1,3-dimethyl 6-carboxyl lumazine fragment, the alanine residue, and the benzene is $C_{18}H_{17}N_6O_4$, one carbon and one nitrogen (cyano −CN or isocyano −NC) less than the molecular formula of compound **1** ($C_{19}H_{17}N_7O_4$). This cyano or isocyano group must be located at the 3″-position of the benzene which is 1,2-disubstituted. A strong HMBC correlation from H-4″ to C-9″ (δ_C 163.3) indicated that the functional group at the 3″-position was an isocyano group. Hence, the planar structure of **1** was determined as shown. The absolute configuration of the alanine residue in **1** was determined using Marfey's method.[28] HPLC analysis of the FDAA derivative of the hydrolysate from **1** gave the same retention time and UV as those of the derivative prepared from an authentic L-alanine. Hence, the absolute configuration of **1** was determined as shown.

The molecular formula of compound **2** was determined as $C_{19}H_{18}N_6O_6$ by HRESIMS analysis, which was 19 Da more than that of compound **1**. The spectroscopic data of **2** were almost identical to those of **1** (Table 1). An HMBC correlation from H-4″ (δ_H 8.54) to C-8″ (δ_C 169.4) (Figure 1) indicated that the functional group at the 4″-position in **2** must be a carboxyl acid group (−COOH), which is 19 unit more than the isocyano group (−NC) in **1**. Compound **2** had the same sign of optical rotation as that of **1**, indicating that **2** should have the same configuration as that of **1**. The absolute configuration of the alanine residue in **2** was also determined by using Marfey's method.[28] Hence, the structure of **2** was determined as shown.

Compound **3** was also obtained as a pale yellow oil. Its molecular formula, $C_{19}H_{18}N_6O_7$, was determined on the basis of HRESIMS, which was 16 Da more than that of compound **2**. Its NMR data (Table 1) were similar to those of **2** except for a different amino acid residue in **3**. In the COSY spectrum of **3** (Figure 1), the methine (δ_H 4.54, H-2′) group showed correlation to a hydroxymethyl (δ_H 3.85 and 4.03, H_2-4′). In the HMBC spectrum of **3** (Figure 1), the hydroxymethyl (H_2-4′) showed correlation to the carbonyl (δ_C 167.9, C-3′), indicating the presence of a serine residue. The absolute configuration of the serine residue in **3** was determined by using Marfey's method.[28] HPLC analysis of the FDAA derivative of the hydrolysate from **3** gave the same UV and same retention time as those of the derivative prepared from authentic L-serine. Further, compound **3** had the same sign of optical rotation as terrelumamide B (a methyl ester of **3**),[29] indicating that **3** should have the same configuration as that of terrelumamide B. Hence, the structure of **3** was determined as shown.

Compound **4** was also obtained as a pale yellow oil. Its molecular formula, $C_{21}H_{21}N_7O_7$, was determined on the basis of HRESIMS. Its NMR data (Table 1) were similar to those of **3** except for a different amino acid residue in **4**. In the COSY spectrum of **4** (Figure 1), the methylene (δ_H 2.28 and 2.09, H_2-4′) group showed correlation to a second methylene (δ_H 2.16−2.18, H_2-5′). In the HMBC spectrum of **4** (Figure 1), H_2-5′ showed correlation to the carbonyl (δ_C 173.6, C-6′) and methine (δ_C 54.3, C-2′), indicating the presence of a glutamine residue. The absolute configuration of the glutamine residue in **4** was determined by using Marfey's method.[28] HPLC analysis of the FDAA derivative of the hydrolysate from **4** gave the same retention time and UV as the derivative prepared from authentic L-glutamine. Further, compound **4** had the same sign of optical rotation as aspergilumamide A (a methyl ester of **4**),[30] indicating **4** should have the same configuration as that of aspergilumamide A. Hence, the structure of **4** was determined as shown.

Compound **5** was obtained as a pale yellow oil. The molecular formula of compound **5** was determined as $C_{21}H_{22}N_6O_7S$ by HRESIMS analysis, which is 14 Da less than that of compound **8** (penilumamide A).[27] The spectroscopic data of **5** (Table 1, Figure 1) were almost the same as those of **8** except for the absence of a methoxy group in **5**. It was readily deduced that **8** was the methyl ester of **5**. In the COSY spectrum of **5** (Figure 1), the H_2-4′ methylene group did not show any correlation to the methine group (H-2′), but the formation of **8** through the methylation of **5** further confirmed the structure elucidation. Hence, the structure of **5** was determined as shown.

Compound **6** was obtained as a colorless powder. The molecular formula of compound **6** was determined as

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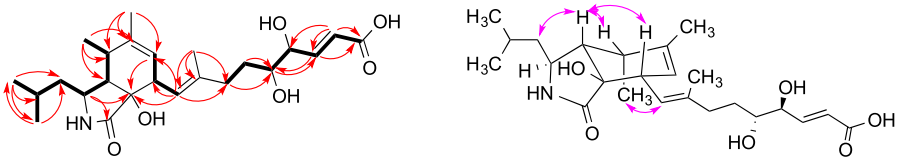

Figure 2. Key COSY (bold), HMBC (arrows, red), and NOESY (double-headed arrows, pink) correlations of **6**.

$C_{24}H_{37}NO_6$ by HRESIMS analysis. The ^{1}HNMR and HSQC spectra showed the presence of five methyl groups, three methylenes, 11 methines including four olefinic and three oxygenated/nitrogenated, and five nonprotonated carbons. In the COSY spectrum of **6**, three spin systems were identified, CH_3−CH(CH_3)−CH_2−CH−CH−CH(CH_3)− [23−22(24)−10−3−4−5−11], =CH−CHCH= (7−8−13), and CH_2−CH_2−CH(O)−CH(O)−CH=CH− (15−16−17−18−19−20) (Figure 2). In the HMBC of **6**, the methyl group at the 12-position (δ_H 1.77) showed correlations to C-5 (δ_C 34.9), C-6 (δ_C 140.8), and C-7 (δ_C 126.2), and the methyl group at the 25-position (δ_H 1.66) correlated to C-13 (δ_C 125.1), C-14 (δ_C 138.0), and C-15 (δ_C 37.0), which connected the three spin systems. Both H-19 (δ_H 7.06) and H-20 (δ_H 6.04) showed HMBC correlations to C-21 (δ_C 170.4) (Table 2), which should

Table 2. NMR Spectroscopic Data (400 MHz) for 6 and 7 in CD_3OD

	6		7		
no.	δ_C	δ_H J (Hz)	no.	δ_C	δ_H J (Hz)
1	178.6		1	166.3	
3	53.0	3.06, m	2	139.0	
4	57.2	2.12, t (5.3)	3	139.9	
5	34.9	2.72 m	4	78.0	5.66, t (4.0)
6	140.8		5	37.8	3.21, m; 2.95, m
7	126.2	5.30, br s	6	57.4	3.67, s
8	45.0	3.10, m	1′	124.6	
9	81.9		2′	130.7	6.47, d (1.8)
10	49.5	1.43, m; 1.39, m	3′	128.1	
11	15.6	1.23, d (7.4)	4′	150.0	
12	20.4	1.77, s	5′	113.8	6.56, d (7.8)
13	125.1	5.50, dd (10.0, 1.1)	6′	128.1	6.56, dd (7.8, 1.8)
14	138.0		1″	125.6	
15	37.0	2.22, m; 2.18, m	2″	129.5	7.54, d (9.0)
16	31.6	1.82, m; 1.51, m	3″	115.7	6.90, d (9.0)
17	74.2	3.60, ddd (9.1, 5.8, 2.0)	4″	159.4	
18	75.5	4.11, ddd (5.8, 4.9, 1.7)	5″	115.7	6.88, d (9.0)
19	149.5	7.06, dd (15.7, 4.9)	6″	129.5	7.51, d (9.0)
20	123.1	6.04 dd (15.7, 1.7)	1‴	27.5	3.13, d (7.8)
21	170.4		2‴	122.5	5.15, m
22	26.2	1.72, m	3‴	131.0	
23	24.5	0.94, d (6.2)	4‴	24.5	1.70, s
24	21.5	0.94, d (6.2)	5‴	16.5	1.64, s
25	16.3	1.66, d (1.1)			

be a carbonyl group (either −COO− or −CONH−) on the basis of its chemical shift. H-3 (δ_H 3.06), H-5 (δ_H 2.72), H-7 (δ_H 5.30), and H-13 (δ_H 5.50) showed HMBC to an sp^3 nonprotonated carbon (δ_C 81.9, C-9), indicating that C-9 must be oxygenated, and it formed a cyclohexene with C-4−C-5−C-6=C-7−C-8. Both H-3 and H-4 (δ_H 2.12) correlated to C-1 (δ_C 178.6), indicating the presence of a γ-lactam (C-3−C-4−C-9−C-1-NH) because of the small chemical shift values of the 3-position (δ_H 3.06/δ_C 53.0) when compared with the corresponding chemical shift values of the 4-position (δ_H 5.66/δ_C 78.0) in γ-lactone in compound **7** (Table 2). The cyclohexene and the γ-lactam were connected at the 4- and 9-positions. Therefore, the planar structure of compound **6** was determined as shown. Compound **6** is very similar to amiaspochalasin F,[31] which is the methyl ester of compound **6**. The relative configuration in the ring system of compound **6** was the same as that of amiaspochalasin F, as evidenced by the NOESY correlations from H-4 to H-10, H-5, and H-8 and from H-13 to H_3-11 (Figure 2). The similar ECD spectrum (Figure 3) and the

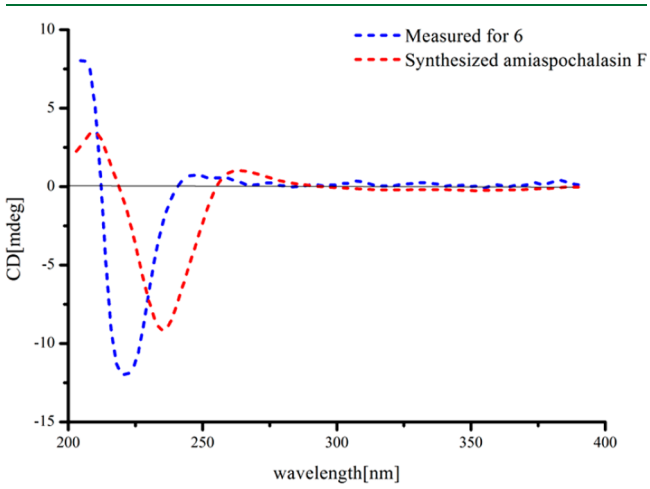

Figure 3. Experimental ECD spectra of **6**.

same sign of optical rotation as those of amiaspochalasin F indicated that **6** should have the same configuration as that of amiaspochalasin F. The formation of amiaspochalasin F through the methylation of **6** further confirmed the structure elucidation of **6**.

Compound **7** was also obtained as a colorless powder. The molecular formula of compound **7** was determined as $C_{23}H_{24}O_5$ by HRESIMS analysis. The UV absorptions at 201, 230, and 305 nm were similar to those of **12**,[32] indicating a butenolide skeleton. The ^{1}H NMR and HSQC spectra of **7** (Table 2) showed the presence of two methyls, one methoxy, two methines, two methylenes, and seven aromatic protons. The COSY spectrum of **7** (Figure 4) indicated the existence of four spin systems, =CH−CH_2, CH−CH_2, a 1,4-disubstituted benzene, and a 1,2,4-trisubstituted benzene. In the HMBC spectrum of **7**, H-5‴ (δ_H 6.88) correlated to C-2‴ (δ_C 122.5), C-

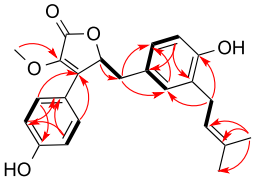

Figure 4. Key COSY (bold) and HMBC (arrows, red) correlations of **7**.

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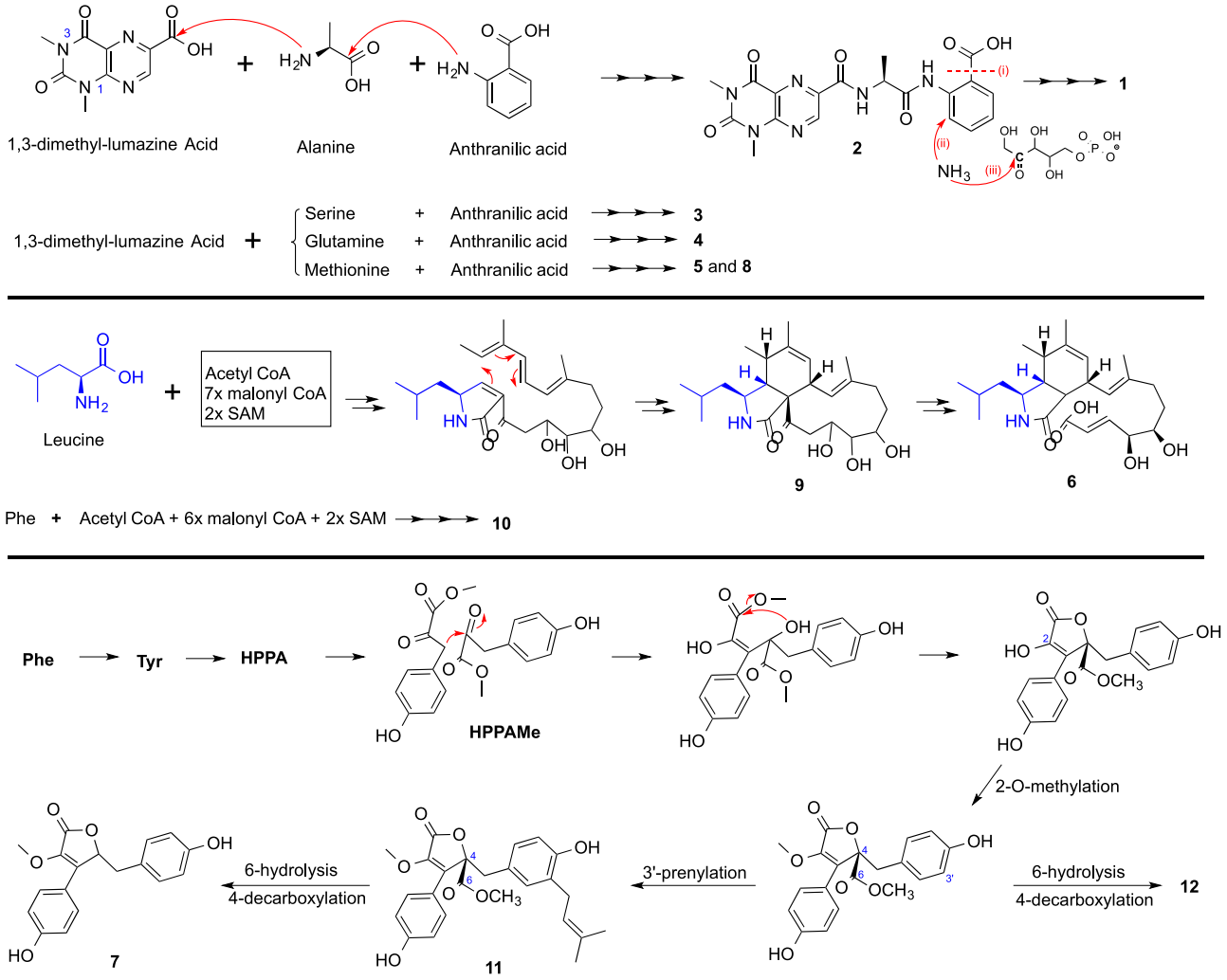
Scheme 1. Plausible Biosynthetic Pathways of Compounds 1−12



3‴ (δ_C 131.0), and C-4‴ (δ_C 24.5), indicating the presence of an isoprenyl group. HMBC correlations from H-1‴ (δ_H 3.13) to C-2′ (δ_C 130.7), C-3′ (δ_C 128.1), and C-4′ (δ_C 150.0) indicated that the isoprenyl group was connected to the 3′-position of the 1,2,4-trisubstituted benzene. The HMBC spectrum of **7** also exhibited correlations from the methoxy (δ_H 3.67) to C-2 (δ_C 139.0), indicating that the α,β-unsaturated α-methoxy γ-lactone was connected to the 1,4-disubstituted benzene (from C-3 to C-1″) and the 1,2,4-trisubstituted benzene (from C-4 to C-1′ via the methylene bridge at the 5-position). The same sign of optical rotation of **7** as that of **12** indicated that **7** should have the same configuration as that of **12**.[33] Hence, the structure of **7** was determined as shown.

In addition to compounds **1**−**7**, seven known compounds, penilumamide (**8**),[27] aspochalasin E (**9**),[34] cytochalasin Z_{20} (**10**),[35] 2-*O*-methylbutyrolactone I (**11**),[36] 2-*O*-methyl-4-(4-hydroxyphenyl)-5-(4-hydroxyphenylmethyl)-2-hydroxyfurane-2-one (**12**),[33] cyclic Pro-Gly-Val-Gly-Tyr(8-OH)-Gly-Trp (**13**),[37] and cyclic Pro-Gly-Val-Gly-Tyr(8-OH, 3-prenyl)-Gly-Trp (**14**),[37] were obtained. The structures of these known compounds were determined on the basis of comparisons of NMR and ESIMS data with previously reported data.

Lumazine peptides are a small class of natural products.[38] So far, only a dozen such compounds have been isolated from natural sources.[29,30,38−41] Biogenetically, compounds **1** and **2** could be derived from lumazine acid, alanine, and anthranilic acid, while **3**, **4**, **5**, and **8**[42] could be derived from lumazine-6-carboxylic acid, serine or glutamine, or methionine sulfoxide, respectively, and anthranilic acid.[27] It is also possible that compound **1** was derived from compound **2** and glycine. Decarboxylation of compound **1** and coupling of glycine to the decarboxylated **1** followed by decarboxylation and oxidation would yield compound **1** (Scheme 1). Compounds **6**, **9**,[34] and **10**[35] belong to the widely distributed cytochalasin or aspochalasin class of fungal metabolites, which are derived from a leucine (or phenyl alanine) and a polyketide [one acetyl CoA, 7 (or 6) malonyl CoA, and 2 *S*-Adenosyl methionines] (Scheme 1).[42] Compounds **7**, **11**,[36] and **12**[33] are γ-lactones, which could be biosynthesized from phenylalanine (Scheme 1).[43]

Compounds **1**−**14** were evaluated for their activity against *S. aureus* and *E. coli*, but none were active at 80 μg/mL. However, in the presence of 20 μM disulfiram, compounds **12**−**14** were active against *S. aureus* with MIC values of 10 μg/mL (Table 3). The combination of disulfiram with other antibacterial agents provides an opportunity to expand a previously untapped bioactive chemical space. Compounds **1**−**14** were further tested for their antiproliferative activity against A2780 (human ovarian cancer cells), but none were active at 40 μM (the highest concentration tested). When evaluated in a mammalian cell-

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Table 3. Activities of Compounds 12−14 against *S. aureus* (ATCC 43300)

	MIC (μg/mL)	
compound	compound alone	+ disulfiram (20 μM)
12	NA[a]	10
13	NA	10
14	NA	10
chloramphenicol	6.25	2.5

[a]NA: not active at 80 μg/mL.

based assay designed to monitor TNF-α-induced NF-κB inhibitory activity, compounds **9** and **13** were found to mediate inhibitory responses with IC_{50} values of 3.1 ± 1.0 and 10.3 ± 2.0 μM, respectively. Thus, in the absence of a cytotoxic response, inhibition of TNF-α-induced NF-κB activity suggests the potential of mediating a cancer chemopreventive response.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations and ECD and FT-IR spectra were measured with a Rudolph Research Analytical autoPol automatic polarimeter, JASCO J-815 ECD, and Thermo Scientific Nicolet iS10 IR spectrometer, respectively. 1D and 2D NMR spectra were recorded on a Bruker AM-400 spectrometer. The 3.35 and 49.3 ppm resonances of CD_3OD and the 2.50 and 39.5 ppm resonances of DMSO-d_6, were used as internal references for ^1H and ^{13}C NMR spectra, respectively. An Agilent 6530 accurate-Mass Q-TOF LC-MS spectrometer was used to record high-resolution mass spectra. Preparative HPLC was carried out on an Ultimate 3000 chromatographic system with a Phenomenex preparative column (Phenyl-Hexyl, 5 μm, 100 × 21.2 mm) and semipreparative HPLC on an Ultimate 3000 chromatographic system with a Phenomenex semipreparative column (C_{18}, 5 μm, 250 × 10 mm), a Dionex Ultimate 3000 DAD detector, and a Dionex Ultimate 3000 automated fraction collector, and all solvents were HPLC grade. Diaion HP-20 was used to run open column chromatography.

Strain Isolation and Fermentation. The strain FS888 was isolated from a soil sample collected at Hanauna Bay (latitude: 21.264978° N; longitude: 157.692057° W), Oahu, Hawaii, in January 2015. The rDNA ITS1-4 region sequence of fungus has been submitted to GenBank (accession number MT256067). The strain was deposited in a −80 °C freezer at Daniel K. Inouye College of Pharmacy, University of Hawaii at Hilo, HI, USA. The strain was grown on PDA plates at 28 °C for 3 days; then it was cut into small pieces and inoculated into 20 L autoclaved sterilized liquid medium [mannitol 20 g, glucose 10 g, monosodium glutamate 5 g, KH_2PO_4 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.3 g, and yeast extract 3 g for 1 L of distilled water; pH 6.5 prior to sterilization] for fermentation at 24 °C for 30 days.

Extraction and Isolation. The fermentation broth of FS888 (20 L) was filtered through cheese cloth to separate the mycelia from the aqueous mycelia. The former was extracted three times with acetone. Acetone was removed under reduced pressure to afford an aqueous mycelia extraction. After combining the aqueous mycelia extraction and supernatant solution, it was separated into four fractions by an HP-20 column eluting with MeOH−H_2O (30, 50, 90, and 100%). Fraction 3 (4.23 g) was separated by prep-HPLC (Phenyl-Hexyl, 100 × 21.20 mm, 5 μ; 8 mL/min) eluted with 40−100% MeOH/H_2O in 20 min to yield 26 subfractions (SFr 3-1−26). SFr 3-17 (170 mg) was purified by semipreparative HPLC (63% MeOH/H_2O, v/v, 3.0 mL/min) over a C_{18} column to afford compounds **1** (4.7 mg, t_R 10.1 min), **7** (3.1 mg, t_R 27.9 min), **10** (1.2 mg, t_R 19.4 min), and **14** (1.9 mg, t_R 21.2 min). SFr 3-9 (210 mg) was purified by semipreparative HPLC (35% MeCN/H_2O, v/v, 1.0% formic acid, 3.0 mL/min) over a C_{18} column to afford compounds **2** (7.2 mg, t_R 24.7 min) and **5** (180.2 mg, t_R 14.1 min). SFr 3-7 (131.1 mg) was purified by semipreparative HPLC (25% MeCN/H_2O, v/v, 1.0% formic acid, 3.0 mL/min) over a C_{18} column to afford compounds **3** (3.7 mg, t_R 15.3 min) and **4** (5.1 mg, t_R 13.2 min). SFr 3-13 (279.1 mg) was purified by semipreparative HPLC (40% MeCN/H_2O, v/v, 1.0% formic acid, 3.0 mL/min) over a C_{18} column to afford compounds **6** (12.2 mg, t_R 30.1 min) and **12** (1.7 mg, t_R 15.6 min). SFr 3-12 (201.2 mg) was purified by semipreparative HPLC (40% MeOH/H_2O, v/v, 1.0% formic acid, 3.0 mL/min) over a C_{18} column to afford compound **13** (2.1 mg, t_R 7.8 min). SFr 3-18 (251.0 mg) was purified by semipreparative HPLC (70% MeOH/H_2O, v/v, 3.0 mL/min) over a C_{18} column to afford compounds **11** (3.0 mg, t_R 14.7 min) and **9** (22.5 mg, t_R 22.5 min). SFr 3-23 (26.5 mg) was purified by semipreparative HPLC (60% MeCN/H_2O, v/v, 1.0% formic acid, 3.0 mL/min) over a C_{18} column to afford compound **8** (8.2 mg, t_R 5.3 min).

*Penilumamide F (**1**):* pale yellow oil; $[\alpha]_D^{25}$ +60 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 232 (2.26), 265 (1.68), 317 (1.47) nm; IR (KBr) ν_{max} 3273, 2945, 2841, 2360, 1652, 1417, 1114, 1012 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HRESIMS *m/z* 408.1419 $[M + H]^+$ (calcd for $C_{19}H_{18}N_7O_4$, 408.1420).

*Penilumamide G (**2**):* pale yellow oil; $[\alpha]_D^{25}$ +52 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 221 (2.38), 250 (4.13), 334 (1.69) nm; IR (KBr) ν_{max} 3316, 2943, 2831, 1448, 1112, 1022 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HRESIMS *m/z* 427.1378 $[M + H]^+$ (calcd for $C_{19}H_{19}N_6O_6$, 427.1366).

*Penilumamide H (**3**):* pale yellow oil; $[\alpha]_D^{25}$ +55 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 221 (2.25), 249 (1.74), 334 (1.01) nm; IR (KBr) ν_{max} 3316, 2943, 2831, 2041, 1449, 1115, 1022 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HRESIMS *m/z* 443.1321 $[M + H]^+$ (calcd for $C_{19}H_{19}N_6O_7$, 443.1315).

*Penilumamide I (**4**):* pale yellow oil; $[\alpha]_D^{25}$ +30 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 223(2.47), 246 (2.13), 334 (1.69) nm; IR (KBr) ν_{max} 3316, 2943, 2831, 2040, 1448, 1114, 1022 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HRESIMS *m/z* 484.1583 $[M + H]^+$ (calcd for $C_{21}H_{22}N_7O_7$, 484.1581).

*Penilumamide J (**5**):* pale yellow oil; $[\alpha]_D^{25}$ +41 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 222 (4.59), 248 (4.44), 336 (3.98) nm; IR (KBr) ν_{max} 3316, 2943, 2831, 1448, 1112, 1022 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HRESIMS *m/z* 503.1355 $[M + H]^+$ (calcd for $C_{21}H_{23}N_6O_7S$, 503.1349).

*Amiaspochalasin I (**6**):* colorless powder; $[\alpha]_D^{25}$ +5.2 (*c* 0.10, MeOH); ECD (MeOH) λ_{max} (Δε) 205 (+7.7), 231 (−3.5) nm; UV (MeOH) λ_{max} (log ε) 206 (4.97) nm; IR (KBr) ν_{max} 3319, 2943, 2831, 1686, 1448, 1410, 1115, 1030 cm^{-1}; ^1H and ^{13}C NMR data, Table 2; HRESIMS *m/z* 436.2698 $[M + H]^+$ (calcd for $C_{24}H_{38}NO_6$, 436.2699).

*4-Demethoxycarbonyl-2-O-methylbutyrolactone I (**7**):* colorless powder; $[\alpha]_D^{25}$ −3.7 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.27), 304 (3.50) nm; IR (KBr) ν_{max} 3320, 1685, 1430, 1112, 1035, 1011 cm^{-1}; ^1H and ^{13}C NMR data, Table 2; HRESIMS *m/z* 381.1624 $[M + H]^+$ (calcd for $C_{23}H_{25}O_5$, 381.1703).

Determination of the Absolute Configurations of 1−4. A solution of compound **1** (**2**, **3**, and **4**, 1.0 mg) in 6.0 M HCl (1.0 mL) was sealed in a vial and heated at 110 °C for 20 h. HCl was removed under vacuum. The hydrolysate was dissolved by 250 μL of H_2O. A 50 μL amount of the hydrolysate was placed in a 1 mL reaction vial and treated with a 1% solution of FDAA (200 μL) in acetone followed by 1.0 M $NaHCO_3$ (40 μL). The reaction mixture was heated at 45 °C for 1.0 h and then was acidified with 2.0 M HCl (20 μL). Separately, the standard amino acids (L-Ala, D-Ala, L-Ser, D-Ser, L-Glu, D-Glu) were derivatized with FDAA in the same manner as that of **1** (or **2** or **3** or **4**). All FDAA derivatives were analyzed by HPLC using MeCN/H_2O (0.01% TFA) as the mobile phase.

Chemical Transformation of 5 and 6. To compound **5** (9.0 mg) were added CH_3OH (100 μL)/toluene (400 μL) and 35 μL of $TMSCHN_2$ at room temperature, and the mixture was stirred for 30 min at room temperature.[29] After removing the solvent, the residue was further purified by HPLC [40% MeCN/H_2O, 3.0 mL/min] over a YMC-pack ODS-A column to afford compound **8** (1.1 mg, t_R = 11.0 min) from **5**. The products of **5** were identified by ESIMS (*m/z* 517.2 $[M + H]^+$, 1055.1 $[2 M + Na]^+$), co-HPLC (Figure S47), and ^1H NMR (Figure S48). To compound **6** (3.0 mg) were added CH_3OH (100 μL)/toluene (350 μL) and 16 μL of $TMSCHN_2$ at room temperature, and the mixture was stirred for 30 min at room temperature.[29] After removing the solvent, the residue was further purified by HPLC [50%

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MeCN/H_2O, 3.0 mL/min] over a YMC-pack ODS-A column to afford the compound amiaspochalasin F (2.5 mg, t_R = 18.8 min) from **6**. The product, amiaspochalasin F, was identified by ESIMS (m/z 450.2 $[M + H]^+$, 921.52 $[2 M + Na]^+$) and ^1H NMR (Figure S49).

Antiproliferative Assays. The viability of A2780 human ovarian cancer cells was determined using the CyQuant assay according to the manufacturer's instructions (Life Technologies, CA, USA). Briefly, cells were cultured in 96-well plates at 1000 cells per well for 24 h and subsequently treated with compounds (20 μg/mL) for 72 h and analyzed. Relative viability of the treated cells was normalized to the DMSO-treated control cells.[44−46] Cisplatin was used as a positive control, which had an IC_{50} value of 0.36 μM. All experiments were performed in triplicate.

NF-κB Assay. We employed HEK 293 from Panomics for monitoring changes occurring along the NF-κB pathway.[47] Stable constructed cells were seeded into 96-well plates at 20×10^3 cells per well. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Co.), supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. After 48 h of incubation, the medium was replaced and the cells were treated with various concentrations of test substances. TNF-α (human, recombinant, *E. coli*, Calbiochem) was used as an activator at a concentration of 2 ng/mL (0.14 nM). The plate was incubated for 6 h. Spent medium was discarded, and the cells were washed once with PBS. Cells were lysed using 50 μL (for 96-well plate) of reporter lysis buffer from Promega, by incubating for 5 min on a shaker, and stored at −80 °C. The luciferase assay was performed using the Luc assay system from Promega. The gene product, luciferase enzyme, reacts with the luciferase substrate, emitting light, which was detected using a luminometer (LUMIstar Galaxy BMG). Data for NF-κB inhibition are expressed as IC_{50} values (i.e., concentration required to inhibit TNF-α-induced NF-κB activity by 50%). As positive controls, two known NF-κB inhibitors were used, TPCK (N_α-tosyl-L-phenylalanine chloromethyl ketone) and BAY-11-7082 (which selectively and irreversibly inhibit NF-κB activation by blocking TNF-α-induced phosphorylation of IκB-α without affecting constitutive IκB-α phosphorylation), yielding IC_{50} values of 5.3 ± 0.9 and 11 ± 1.8 μM, respectively. All experiments were performed in triplicate.

SRB Assay. In order to assess the potential of mediating a cytotoxic response, the cells were treated under the same experimental conditions with each test compound at a concentration of 50 μM, and cell survival was determined by the sulforhodamine B (SRB) assays. After incubation of HEK 293 cells with test compounds, cells were fixed with 10% trichloroacetic acid solution for 30 min and stained with 0.4% SRB in 1% acetic acid solution for 30 min. Protein-bound SRB was dissolved in 10 mM Tris buffer (pH 10.0), and the absorbance was measured at 515 nm. The effect of compounds on cell survival was demonstrated as percentage survival in comparison with vehicle (DMSO)-treated control cells.

Antibacterial Assay. The antibacterial assay was conducted by the previously described method.[48−50] Additionally samples were applied along with the antibiotic adjuvant [disulfiram for *S. aureus*] with their final concentration at 20 μM. DMSO [5%] as well as antibiotic adjuvents [20 μM] was used as negative controls, whereas chloramphenicol was used as a positive control, which was active against *S. aureus* and *E. coli* with MIC values of 6.25 and 5 μg/mL, respectively. The maximum concentration of the used compounds was 80 μg/mL. All experiments were repeatedly performed in triplicate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c00344.

HRESIMS and NMR for compounds **1**−**7**; determination of the absolute configuration of Ala unit in compounds **1** and **2**, Ser unit in compound **3**, and Glu unit in compound **4**; the co-HPLC profiles of synthetic **8** and natural **8**; comparison of the ^1H NMR spectrum of synthetic **8** and natural **8** in CD_3OD; and comparison of the ^1H NMR spectrum of compound **6** and amiaspochalasin F in CD_3OD (PDF)

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Notes

The authors declare no competing financial interest.

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