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Fuscasins A−D, Cycloheptapeptides from the Marine Sponge *Phakellia fusca*

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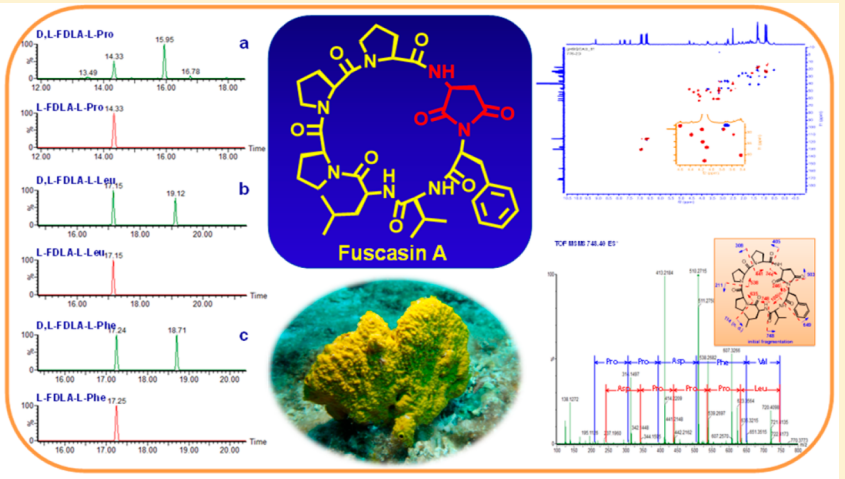
Supporting Information



ABSTRACT: Four new cycloheptapeptides, fuscasins A−D (**1**−**4**), were isolated from the marine sponge *Phakellia fusca* collected from the South China Sea. Their planar structures were fully characterized by spectroscopic methods, and the absolute configurations of amino acid residues were determined using the advanced Marfey's method. Structurally, **1** is a unique cycloheptapeptide with a backbone bearing a pyrrolidine-2,5-dione unit. Among the isolated compounds, **1** exhibited potent growth-inhibitory activity against HepG2 cells with an IC_{50} value of 4.6 μM, whereas it did not show apparent inhibitory effects against the other five human cancer cell lines, MCF-7, HeLa, NCI-H460, PC9, and SW480. Encouragingly, **1** exhibited no cytotoxicity against nonmalignant cells even with a concentration up to 100 μM. These findings suggest that **1** may display a selective inhibitory effect on the growth of HepG2 cells.

Ever since the discovery of spongouridine and spongothymidine, marine sponges have been in the spotlight of natural product chemists for drug discovery.[1] According to the literature, 47% of marine natural products have been isolated from sponges,[2] and most have drug-like properties.[3] Remarkably, sponge-derived cyclic peptides have been actively investigated as biochemical tools and therapeutic agents, due to their significant structural diversity, excellent stability, and effective biological activity.[4,5] Among these, the phakellistatins, mainly isolated from the sponge genus *Phakellia*, represent proline-rich compounds usually containing more than six or seven amino acid residues.[6] Since the isolation of phakellistatin 1[7a] from *Phakellia costata* in the early 1990s, more than 20 phakellistatins have been reported in the past two decades. The significant cytotoxicity of phakellistatins not only provides important evidence for the role of proline residues but also has stimulated the interest of synthetic chemists.[7m,8] Phakellistatins 1−3[7a−c] and phakellistatins 7−10[7g,h] in this series have been synthesized based on their strong cytotoxicity toward P388 murine leukemia cells.[9]

Herein, we report the isolation and characterization of four new cycloheptapeptides containing proline residues, fuscasins A−D (**1**−**4**), from *Phakellia fusca* collected from Yongxing Island in the South China Sea. Their structures were deduced by extensive LC-MS analyses, 1D and 2D NMR spectroscopy, and characterization of degradation products. The cytotoxicities of these compounds were measured against a number of cancer cell lines (MCF7, HeLa, NCI-H460, PC9, HepG2, and

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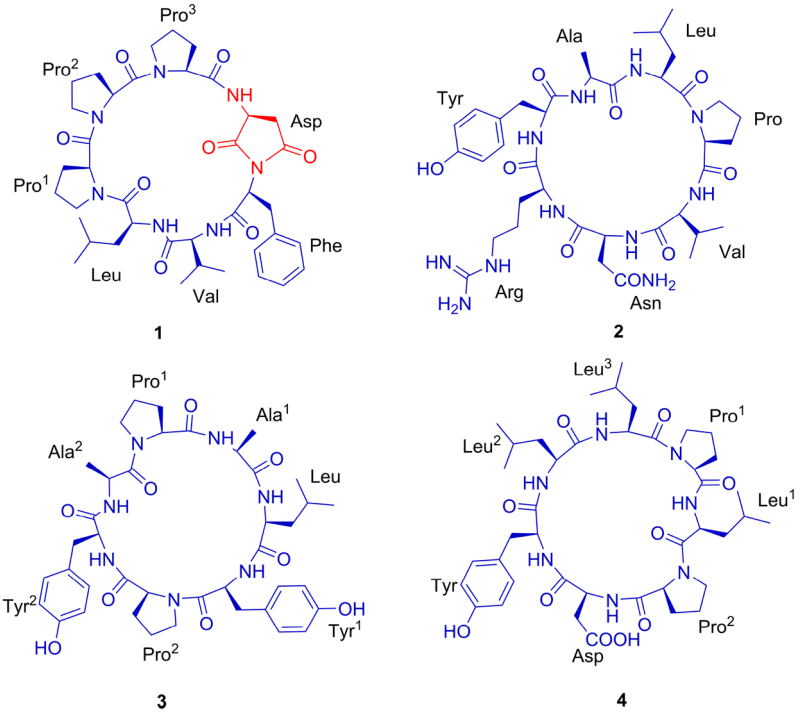
Chart 1



SW480), and **1** exhibited selective cytotoxicity against HepG2 cells.

RESULTS AND DISCUSSION

Fuscasin A (**1**) was obtained as a yellowish, amorphous powder and had a molecular formula of $C_{39}H_{53}N_7O_8$ based on HRESIMS data (m/z 748.4034 $[M + H]^+$) accounting for 17 degrees of unsaturation. The IR spectrum of **1** showed strong absorption bands at 1714 and 1634 cm^{-1} (Figure S10), revealing the presence of amide functionalities. The ^1H NMR spectrum showed three deshielded NH proton signals (δ_H 7.57, 7.90, and 9.13) and seven α-H (δ_H 3.88−4.71), indicating a peptide structure bearing seven residues, while the ^{13}C NMR spectrum revealed the presence of eight carbonyls (δ_C 166.7−173.6) and seven α-amino acid carbon resonances (δ_C 57.7−61.0) (Table 1). Additionally, TOCSY experiments allowed the assignment of seven spin systems, consisting of one Leu, one Val, one Phe (absence of the NH proton), one Asp, and three Pro residues. Amino acid side chains were assigned using COSY, HMBC, and TOCSY NMR spectra whereby connectivity was determined using HMBC and ROESY analysis. HMBC correlations from Leu-NH to Val-CO, from Val-Hα to Phe-CO, from Phe-Hα to Asp-CO, and from Asp-Hα to Pro^3-CO established the five amino acid sequence Pro^3-Asp-Phe-Val-Leu. ROESY correlations from Pro^3-Hα and Pro^1-Hα to Pro^2-Hα and from Pro^1-Hδ to Leu-Hα resulted in the final sequence of the heptapeptide being assigned as *cyclo*-(Pro^1-Pro^2-Pro^3-Asp-Phe-Val-Leu) (Figure 1). The assignments were further confirmed by ESIMS/MS spectra, which gave a series of "b" fragment ions at m/z 649, 503, 405, 308, and 211, corresponding to the successive loss of Val, Phe, Asp, Pro, and Pro (Figure 2). Accordingly, "y" fragment ions at m/z 635, 538, 441, 344, and 246 suggested the successive loss of Leu, Pro, Pro, Pro, and Asp. Because **1** had 17 degrees of unsaturation and seven amino acid residues and the macrocycle simply accounted for 16 degrees of unsaturation, **1** should bear another ring. Further, an HMBC correlation of Phe-Hα with the γ carbonyl carbon of Asp indicated that the γ carbon of Asp was connected to the amino group of the Phe residue. Based on the above evidence, **1** contains a pyrrolidine-2,5-dione unit formed by a condensation between the Asp side chain carboxyl group and the adjacent Phe nitrogen, which results in a unique cycloheptapeptide backbone.[10] The $\Delta\delta_{C\beta\text{-}C\gamma}$ values of the Pro residue (5.2, 10.1, and 8.3 for Pro^1, Pro^2, and Pro^3, respectively) were indicative of one *trans* and two *cis* geometries for the respective proline amide bonds in **1**.[11] The absolute configurations of the amino acid units in **1** were determined by the advanced Marfey's method.[12] The hydrolysis products (6 N HCl, 110 °C, 12 h) of **1** were subjected to L-FDLA derivatization and analyzed by UPLC-ESI-QTOF MS, which showed that all amino acid residues were L-configurations (Figure 3).

Fuscasin B (**2**) was isolated as a yellowish, amorphous powder, and HRESIMS gave an $[M + H]^+$ ion at m/z 814.4574, indicating a molecular formula of $C_{38}H_{59}N_{11}O_9$ and requiring 15 degrees of unsaturation. The peptidic nature of **2** was evident from the abundance of signals in the amide NH region (δ_H 7.34−8.88), from the seven α-H (δ_H 3.68−4.36) in the ^1H NMR spectrum, and from the eight carbonyl carbons (δ_C 170.4−172.8) attributable to amide functionalities in its ^{13}C NMR spectrum (Table 2). TOCSY experiments revealed seven side chain spin systems, consisting of Pro, Val, Asn, Arg, Tyr, Ala, and Leu residues. In analogy to **1**, the linkages and assignments of the amino acid residues in **2** were established by HMBC and ROESY NMR data (Table 2). Two fragments, Ala-Leu and Pro-Val-Asn-Arg, were indicated by the HMBC correlations from Leu-NH to Ala-CO, from Arg-NH to Asn-CO, from Asn-NH to Val-CO, and from Val-NH to Pro-CO. ROESY correlations from Leu-Hα to Pro-Hα, from Ala-NH to Tyr-NH, and from Tyr-NH to Arg-NH resulted in the final

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Table 1. NMR Spectroscopic Data for Fuscasin A (1) at 600 MHz (^{1}H) and 150 MHz (^{13}C) in DMSO-d_6

residue	position	δ_C, type	δ_H, mult (J, Hz)	TOCSY	HMBC (H→C)	ROESY[a]
Pro^1	CO	169.8, C				
	α	58.4, CH	4.40, m	Pro^1-β, γ, δ		Pro^2-α
	β	29.2, CH_2	a: 2.14, m	Pro^1-α, γ, δ	Pro^1-CO	
			b: 1.60[b], dt (13.7, 7.0)	Pro^1-α, γ, δ	Pro^1-CO	Pro^2-α
	γ	24.0, CH_2	a: 1.75[b], m	Pro^1-α, β, δ	Pro^1-Cα	
			b: 1.67, m	Pro^1-α, β, δ	Pro^1-Cα, β	
	δ	46.9, CH_2	a: 3.43,[b] m	Pro^1-α, β, γ		Leu-α
			b: 3.26, m	Pro^1-α, β, γ	Pro^1-Cγ	Leu-α
Pro^2	CO	169.2, C				
	α	57.8, CH	4.65, m	Pro^2-β, γ, δ	Pro^2-Cγ	Pro^1-α, β, Pro^3-α
	β	31.1, CH_2	a: 2.18, m	Pro^2-α, γ, δ	Pro^2-CO	
			b: 1.83,[b] m	Pro^2-α, γ, δ	Pro^2-CO, Cδ	
	γ	21.0, CH_2	a: 1.83,[b] m	Pro^2-α, β, δ	Pro^2-Cδ	
			b: 1.78,[b] m	Pro^2-α, β, δ		
	δ	46.4, CH_2	a: 3.46,[b] m	Pro^2-α, β, γ	Pro^2-Cα	
			b: 3.40,[b] m	Pro^2-α, β, γ		
Pro^3	CO	171.5, C				
	α	57.9, CH	4.42, d (6.4)	Pro^3-β, γ, δ	Pro^3-Cβ, γ, δ	Pro^2-α, Asp-NH
	β	30.2, CH_2	a: 2.09, m	Pro^3-α, γ, δ	Pro^3-CO	
			b: 2.02, m	Pro^3-α, γ, δ		
	γ	21.9, CH_2	a: 1.83,[b] m	Pro^3-α, β, δ		
			b: 1.75,[b] m	Pro^3-α, β, δ		
	δ	46.4, CH_2	a: 3.46,[b] m	Pro^3-α, β, γ		
			b: 3.40,[b] m	Pro^3-α, β, γ		
Asp	CO	173.6, C				
	α	48.6, CH	4.31, m	Asp-β, NH	Pro^3-CO	
	β	35.2, CH_2	a: 2.98,[b] m	Asp-α, NH	Asp-CO, γ-CO	Phe-α
			b: 2.57, dd (17.4, 6.0)	Asp-α, NH	Asp-CO, Cα, γ-CO	
	γ-CO	173.5, C				
	NH		9.13, s	Asp-α, β		Pro^3-α
Phe	CO	166.7, C				
	α	55.4, CH	4.71, m	Phe-β	Asp-CO, γ-CO, Phe-CO, Cβ, 1	Phe-2, 6, Asp-β, Val-NH
	β	34.0, CH_2	a: 3.51, m	Phe-α	Phe-CO, Cα, 1−3, 5−6	Val-NH
			b: 2.99,[b] m	Phe-α	Phe-CO, Cα, 1−3, 5−6	
	1	137.1, C				
	2, 6	129.1, CH	7.43, d (7.5)	Phe-3−5	Phe-Cβ, 2, 4, 6	Phe-α
	3, 5	128.5, CH	7.28, t (7.5)	Phe-2, 4, 6	Phe-C1, 3, 5	
	4	126.8, CH	7.20, t (7.8)	Phe-2, 3, 5, 6	Phe-C2, 6	
Val	CO	169.6, C				
	α	61.0, CH	3.88, t (8.6)	Val-β, γ, γ', NH	Phe-CO, Val-CO, Cβ, γ, γ'	
	β	29.8, CH	1.95, q (7.1)	Val-α, γ, γ', NH	Val-Cα, γ, γ'	
	γ	19.2, CH_3	0.73, d (6.7)	Val-α, β, NH	Val-Cα, β, γ'	
	γ'	19.0, CH_3	0.70, d (6.6)	Val-α, β, NH	Val-Cα, β, γ	
	NH		7.57, d (9.1)	Val-α, β, γ, γ'		Phe-α, β, Leu-NH
Leu	CO	167.2, C				
	α	47.7, CH	4.69, m	Leu-β, γ, δ, δ', NH		Pro^1-δ
	β	40.9, CH_2	a: 1.60[b], dt (13.7, 7.0)	Leu-α, γ, δ, δ', NH	Leu-CO, Cα, γ, δ, δ'	
			b: 1.34, m	Leu-α, γ, δ, δ', NH	Leu-Cα, γ, δ, δ'	
	γ	24.1, CH	1.51, m	Leu-α, β, δ, δ', NH	Leu-Cα, β, δ, δ'	
	δ	22.7, CH_3	0.88, d (3.9)	Leu-α, β, γ, NH	Leu-Cβ, γ, δ'	
	δ'	22.8, CH_3	0.89, d (3.7)	Leu-α, β, γ, NH	Leu-Cβ, γ, δ	
	NH		7.90, d (9.0)	Leu-α, β, γ, δ, δ'	Val-CO	Val-NH

[a]Sequential NOEs. [b]Overlapping signals.

sequence of the heptapeptide being assigned as *cyclo*-(Pro-Val-Asn-Arg-Tyr-Ala-Leu) (Figure 1), which was subsequently confirmed using MS/MS experiments (Figure S19). The $\Delta\delta_{C\beta\text{-}C\gamma}$ value of the Pro residue (9.4) was indicative of a *cis* geometry for the proline amide bond in **2**.[11] The absolute configurations of the amino acid residues in **2** were determined as L-configurations (Figure S20) by advanced Marfey's method.[12]

Fuscasin C (**3**) was isolated as a yellowish, amorphous powder, and HRESIMS data (m/z 776.3983 $[M + H]^+$) supported a molecular formula of $C_{40}H_{53}N_7O_9$, requiring 18 degrees of unsaturation. The ^{1}H and ^{13}C NMR data (Table 3)

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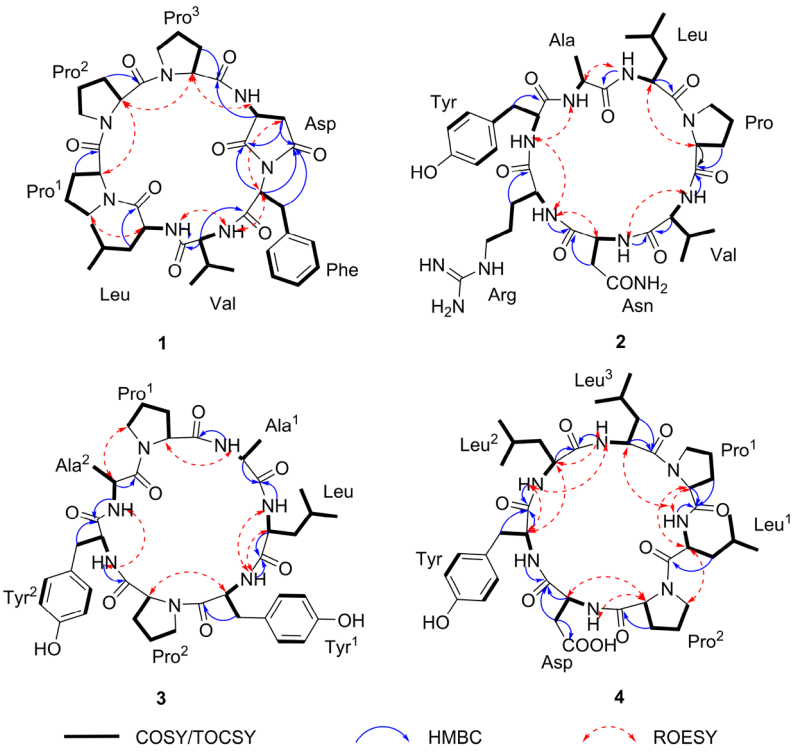

Figure 1. Key COSY/TOCSY, HMBC, and ROESY correlations of **1**−**4**.

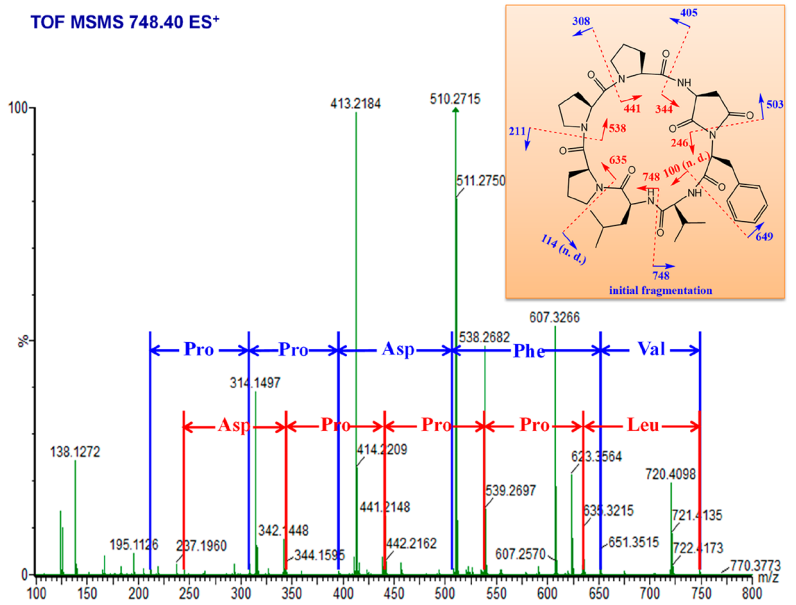

Figure 2. MS/MS fragmentation pattern of **1**. The dashed lines through the structures indicate the "y" and "b" fragments (y shown in red, b shown in blue). Arrows represent direction of sequential fragmentation after initial ring opening during positive-mode MS/MS, and the numbers indicate the corresponding m/z value (n.d.: not detected).

revealed typical peptide characteristic signals: five deshielded NH protons (δ_H 7.46−9.12), seven α-H (δ_H 3.43−4.59), and seven amide carbonyls (δ_C 169.7−171.6). Additionally, TOCSY experiments revealed seven side chain spin systems, consisting of one Leu, two Ala, two Pro, and two Tyr residues. The amino acid side chains were assigned using COSY, HMBC, and TOCSY NMR spectra whereby connectivity was delineated using HMBC, ROESY, and ESIMS/MS analysis. HMBC correlations from Ala^1-NH to Pro^1-CO, from Leu-NH to Ala^1-CO, and from Tyr^1-NH to Leu-CO established the four amino acid sequence Pro^1-Ala^1-Leu-Tyr^1. HMBC correlations from Tyr^2-NH to Pro^2-CO and from Ala^2-NH to Tyr^2-CO established the three amino acid sequence Pro^2-Tyr^2-Ala^2. ROESY correlations from Tyr^1-Hα to Pro^2-Hα and from Pro^1-Hδ to Ala^2-Hα resulted in the final sequence of the heptapeptide being assigned as *cyclo*-(Pro^1-Ala^1-Leu-Tyr^1-

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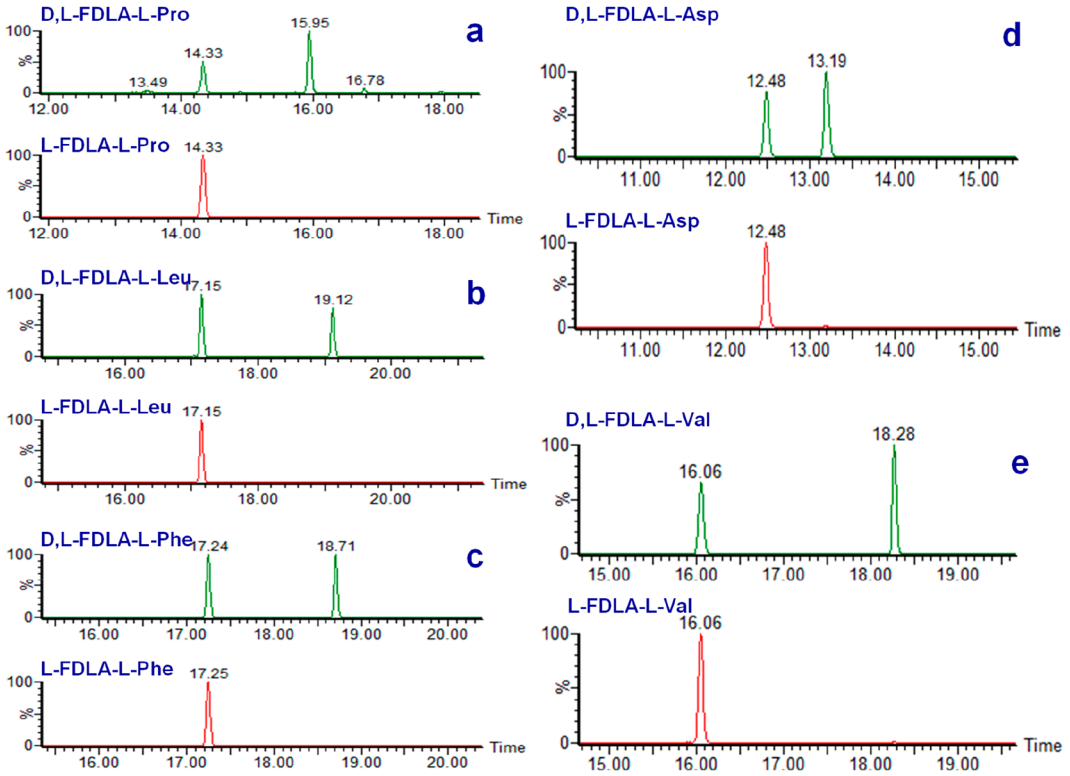

Figure 3. Advanced Marfey's analysis of compound **1** by LC-MS. Selective ion monitoring channels were set up as *m/z* 410 (a), *m/z* 426 (b), *m/z* 460 (c), *m/z* 428 (d), and *m/z* 412 (e). Marfey's derivatives of **1** are shown in red peaks, and standards in green.

Pro^2-Tyr^2-Ala^2) (Figure 1), which was subsequently confirmed using MS/MS experiments (Figure S31). The $\Delta\delta_{C\beta\text{-}C\gamma}$ values of the Pro residues (2.4 and 9.4 for Pro^1 and Pro^2, respectively) were indicative of a *trans* and a *cis* geometry for the respective proline amide bonds in **3**.[11] The absolute configurations of the amino acid residues in **3** were determined as L-configurations (Figure S32) via the advanced Marfey's method.[12]

Fuscasin D (**4**) was isolated as a yellowish, amorphous powder. The HRESIMS data showed an $[M + H]^+$ ion at *m/z* 812.4567, which was suggestive of a molecular formula of $C_{41}H_{61}N_7O_{10}$, establishing 15 degrees of unsaturation. The ^{1}H and ^{13}C NMR data (Table 4) showed typical peptide characteristic signals: five deshielded NH protons (δ_H 7.17−9.03), seven α-H (δ_H 3.99−4.58), and seven amide carbonyls (δ_C 168.8−172.1). Detailed analysis of the 2D NMR (HSQC, TOCSY, and HMBC) spectra of **4** allowed the assignment of seven spin systems, including those of one Tyr, one Asp, two Pro, and three Leu residues. The peptide sequence and connectivity of amino acid residues were determined by HMBC, ROESY, and ESIMS/MS analysis. HMBC correlations from Tyr-NH to Asp-CO, from Leu^2-NH to Tyr-CO, and from Leu^3-NH to Leu^2-CO established the four amino acid sequence Asp-Tyr-Leu^2-Leu^3. HMBC correlations from Leu^1-NH to Pro^1-CO established the two amino acid sequence Pro^1-Leu^1. ROESY correlations from Pro^2-Hα to Asp-Hα, from Asp-NH and Pro^2-Hδ to Leu^1-Hα, and from Leu^1-NH to Leu^3-Hα resulted in the final sequence of the heptapeptide being assigned as *cyclo*-(Pro^1-Leu^1-Pro^2-Asp-Tyr-Leu^2-Leu^3) (Figure 1), which was subsequently confirmed using MS/MS experiments (Figure S43). The $\Delta\delta_{C\beta\text{-}C\gamma}$ values of the Pro residues (9.2 and 4.2 for Pro^1 and Pro^2, respectively) were indicative of a *cis* and a *trans* geometry for the respective proline amide bonds in **4**.[11] The amino acid residues in **4** were determined as L-form amino acids (Figure S44) using the advanced Marfey's method.[12]

The cytotoxicities of **1**−**4** were tested against six human cancer cell lines, MCF-7, HeLa, NCI-H460, PC9, HepG2, and SW480. As a result, **1** exhibited notable cytotoxicity against HepG2 cells with an IC_{50} value of 4.6 μM (Figure S47), and the remaining isolated compounds proved to be inactive at concentrations up to 20 μM (Figure S48). Compound **1** showed no cytotoxicity against the nonmalignant rat cardiomyoblast H9C2 cell line even at concentrations up to 100 μM (Figure S49).

In conclusion, fuscasins A−D (**1**−**4**) are new cycloheptapeptides from the sponge genus *Phakellia* related to the phakellistatin family, which emphasize the unique structural features and wide range of biological properties of cyclopeptides. Structurally, phakellistatins commonly consist of seven amino acid residues, including at least one proline residue. Compounds **1**−**4** are in line with this structural feature. Here we report a new cycloheptapeptide, fuscasin A, which incorporates a pyrrolidine-2,5-dione into the peptide backbone. This enriches the structural diversity of the phakellistatin family. Some phakellistatins show cancer cell cytotoxicities, but there is no obvious regularity based on the structures. Surprisingly, **1** exhibits growth-inhibitory activity selectively against the human cancer cell line HepG2 and shows no cytotoxicity against nonmalignant cells, which suggests that it has potential to be a targeted antitumor drug.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured using a PerkinElmer model 341 polarimeter with a 10 cm

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Table 2. NMR Spectroscopic Data for Fuscasin B (2) at 600 MHz (^{1}H) and 150 MHz (^{13}C) in DMSO-d_6

residue	position	δ_C, type	δ_H, mult (J, Hz)	TOCSY	HMBC (H→C)	ROESY[a]
Pro	CO	170.4, C				
	α	60.4, CH	4.26, m	Pro-β, γ, δ	Pro-CO, Cβ, γ, δ	Leu-α, β, δ′
	β	30.8, CH_2	a: 2.34, dd (12.2, 6.4)	Pro-α, γ, δ	Pro-CO, Cα	
			b: 1.97, m	Pro-α, γ, δ	Pro-CO	
	γ	21.5, CH_2	a: 1.85, dt (13.6, 6.6)	Pro-α, β, δ		
			b: 1.48, m	Pro-α, β, δ		
	δ	46.1, CH_2	a: 3.43, m	Pro-α, β, γ		
			b: 3.31, d (11.2)	Pro-α, β, γ		
Val	CO	170.7, C				
	α	62.6, CH	3.71, t (8.4)	Val-β, γ, γ′, NH	Val-CO, β, γ, γ′	Asn-α
	β	29.5, CH	2.13, dq (14.6, 6.8)	Val-α, γ, γ′, NH		Val-NH
	γ	19.4, CH_3	0.91, d (6.5)	Val-α, β, NH	Val-α, β, γ′	
	γ′	19.5, CH_3	0.93, d (7.0)	Val-α, β, NH	Val-α, β, γ	
	NH		8.12, d (8.0)	Val-α, β, γ, γ′	Pro-CO	Asn-α, NH, Leu-α
Asn	CO	172.8, C				
	α	49.6, CH	4.32, m	Asn-β, NH		Val-α, NH, Arg-NH
	β	36.3, CH_2	a: 3.18, m	Asn-α, NH	Asn-γ-CO	Arg-α, NH
			b: 3.06, dd (14.0, 3.4)	Asn-α, NH	Asn-CO	Arg-α
	γ-CO	171.22				
	NH		7.62, d (5.7)	Asn-α, β	Val-CO	Val-NH, γ-NH_2
	γ-NH_2		a: 7.82, s			
			b: 7.34, d (6.6)			Val-NH
Arg	CO	170.8, C				
	α	55.4, CH	3.68, p (4.1)	Arg-β, γ, δ, NH	Arg-CO	Asn-β, Leu-δ′
	β	27.2, CH_2	a: 1.49, m	Arg-α, γ, δ		
			b: 1.47,[b] m	Arg-α, γ, δ		
	γ	23.7, CH_2	a: 1.24, m	Arg-α, β, δ		
			b: 0.98, m	Arg-α, β, δ		
	δ	40.4, CH_2	a: 2.87[b], d (17.8)	Arg-α, β, γ		
			b: 2.87,[b] d (17.8)	Arg-α, β, γ		
	guanidine	157.5, C				
	NH		8.83, d (8.9)	Arg-α, β, γ, δ	Asn-CO	Tyr-NH, Asn-α, β
Tyr	CO	171.9, C				
	α	55.3, CH	4.27, m	Tyr-β		Tyr-β, 2, 6
	β	36.0, CH_2	a: 3.00, dd (14.0, 3.4)	Tyr-α	Tyr-CO	
			b: 2.74, t (13.0)	Tyr-α	Tyr-Cα, 1, 2, 6	
	1	128.5, C				
	2, 6	129.8, CH	6.89, d (8.2)	Tyr-3, 5	Tyr-Cβ, 2, 4, 6	
	3,5	115.0, CH	6.62, d (8.0)	Tyr-2, 6	Tyr-C1, 3−5	
	4	155.9, C				
	NH		8.04, d (9.5)	Tyr-α, β		Ala-α, NH, Tyr-2, 6
Ala	CO	172.3, C				
	α	48.1, CH	4.36, t (17.8)	Ala-β, NH	Ala-CO, Cβ	Leu-NH, Tyr-NH
	β	16.2, CH_3	1.07, d (6.4)	Ala-α, NH	Ala-CO, Cα	
	NH		7.34, d (6.6)	Ala-α, β		Tyr-NH
Leu	CO	170.9, C				
	α	51.4, CH	4.15, m	Leu-β, γ, δ, δ′, NH		Pro-α, Val-NH
	β	38.2, CH_2	a: 1.58, ddd (14.6, 11.9 3.7)	Leu-α, γ, δ, δ′, NH		
			b: 1.21, m	Leu-α, γ, δ, δ′, NH		Pro-α
	γ	24.3, CH	1.75, m	Leu-α, β, δ, δ′, NH		
	δ	23.4, CH_3	0.87, d (6.6)	Leu-α, β, γ, NH	Leu-Cβ, γ, δ′	
	δ′	20.7, CH3	0.78, d (6.4)	Leu-α, β, γ, NH	Leu-Cβ, γ, δ	Pro-α, Arg-α
	NH		8.88, d (4.7)	Leu-α, β, γ, δ, δ′	Ala-CO	Ala-α

[a]Sequential NOEs. [b]Overlapping signals.

length cell at room temperature. The UV spectra were recorded on a Hitachi U-3010 spectrophotometer, and the IR spectra were collected on a Bruker Tensor 27 FT-IR instrument with an ATR accessory. NMR experiments were conducted on an Agilent 600 MHz NMR instrument, where chemical shifts (δ) are referenced to the DMSO-d_6 residual solvent signal (δ_H 2.50, δ_C 39.52). HRESIMS and ESIMS/MS spectra were acquired with a Waters Xevo G2-XS QTOF spectrometer. UPLC-MS spectra were obtained using a Waters Acquity UPLC system equipped with a Waters XevoG2-XS QTOF spectrometer and a C18 Acquity UPLC HSST3 column (Waters, 2.1 × 100 mm, 1.8 μm). Preparative medium-pressure liquid chromatography (MPLC) was carried out on an Interchim PuriFlash 450

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Table 3. NMR Spectroscopic Data for Fuscasin C (3) at 600 MHz (^{1}H) and 150 MHz (^{13}C) in DMSO-d_6

residue	position	δ_C, type	δ_H, mult (J, Hz)	TOCSY	HMBC (H→C)	ROESY[a]
Pro^1	CO	171.3, C				
	α	62.5, CH	4.13, m	Pro^1-β, γ, δ	Pro^1-CO, Cβ	Ala^2-NH
	β	28.6, CH$_2$	a: 2.21, m	Pro^1-α, γ, δ	Pro^1-Cδ	
			b: 1.77, m	Pro^1-α, γ, δ	Pro^1-CO, Cβ	Ala^1-NH
	γ	25.0, CH$_2$	a: 2.03, m	Pro^1-α, β, δ	Pro^1-Cα	
			b: 1.86, m	Pro^1-α, β, δ		
	δ	46.6, CH$_2$	a: 3.72, t (8.6)	Pro^1-α, β, γ		Ala^2-α
			b: 3.68, m	Pro^1-α, β, γ		
Ala^1	CO	171.22, C				
	α	48.5, CH	4.18,[b] m	Ala^1-β, NH	Ala^1-CO, Cβ	Leu-α
	β	16.8, CH$_3$	1.30, d (7.3)	Ala^1-α, NH	Ala^1-CO, Cα	Leu-NH
	NH		7.70, d (7.2)	Ala^1-α, β	Pro^1-CO, Ala^1-Cα, β	Leu-NH, Pro^1-β
Leu	CO	171.16, C				
	α	50.8, CH	4.38, dt (10.7, 5.0)	Leu-β, γ, δ, δ', NH	Leu-CO	Tyr^1-NH, Ala^1-α
	β	40.1, CH$_2$	a: 1.68, m	Leu-α, γ, δ, δ', NH	Leu-Cδ	
			b: 1.25, m	Leu-α, γ, δ, δ', NH	Leu-CO, Cγ, δ	
	γ	24.4, CH	1.56, m	Leu-α, β, δ, δ', NH	Leu-Cδ, δ'	
	δ	21.8, CH$_3$	0.93,[b] d (6.5)	Leu-α, β, γ, NH	Leu-Cβ, γ, δ'	
	δ'	23.2, CH$_3$	0.87, d (6.7)	Leu-α, β, γ, NH	Leu-Cβ, γ, δ	
	NH		7.46, d (6.2)	Leu-α, β, γ, δ, δ'	Leu-Cα, Ala^1-CO	Tyr^1-α, NH, Ala^1-β, NH
Tyr^1	CO	170.1, C				
	α	54.0, CH	4.16,[b] m	Tyr^1-β		Leu-NH, Tyr^1-2, 6, Pro^2-α
	β	35.6, CH$_2$	a: 2.88, dd (14.0, 6.1)	Tyr^1-α	Tyr^1-CO, Cα, 1, 2, 6	Tyr^1-2, 6, NH
			b: 2.76, m	Tyr^1-α	Tyr^1-CO, Cα, 1, 2, 6	Tyr^1-2,6,NH
	1	126.0, C				
	2, 6	129.7, CH	6.94, d (8.1)	Tyr^1-3, 5	Tyr^1-Cβ, 2−6	Tyr^1-α, β, NH
	3,5	115.3, CH	6.69, d (8.2)	Tyr^1-2, 6	Tyr^1-C1, 3, 4, 5	
	4	156.1, C				
	NH		9.12, d (4.1)	Tyr^1-α, β	Leu-CO	Leu-α, NH, Tyr^1-β, 2, 6
Pro^2	CO	169.7, C				
	α	60.0, CH	3.43, d (7.8)	Pro^2-β, γ, δ	Pro^2-CO, Cβ, γ, δ	Tyr^1-α
	β	29.9, CH$_2$	a: 1.64, m	Pro^2-α, γ, δ	Pro^2-CO, Cδ	
			b: 0.93,[b] d (6.5)	Pro^2-α, γ, δ	Pro^2-CO, Cα	
	γ	20.5, CH$_2$	a: 1.31,[b] d (6.8)	Pro^2-α, β, δ	Pro^2-Cα	
			b: 0.52, m	Pro^2-α, β, δ		
	δ	45.6, CH$_2$	a: 3.05,[b] m	Pro^2-α, β, γ		Tyr^2-α
			b: 2.71, m	Pro^2-α, β, γ		
Tyr^2	CO	170.3, C				
	α	56.5, CH	4.08, m	Tyr^2-β	Tyr^2-CO	Pro^2-δ, Tyr^2-2, 6
	β	36.5, CH$_2$	a: 3.06,[b] m	Tyr^2-α	Tyr^2-C1, 2, 6	
			b: 2.73, d (3.3)	Tyr^2-α	Tyr^2-CO, Cα, 1, 2, 6	Tyr^2-2, 6
	1	127.1, C				
	2, 6	129.3, CH	6.89, d (8.1)	Tyr^2-3, 5	Tyr^2-Cβ, 2−6	Tyr^2-α, β, NH
	3,5	114.9, CH	6.64, d (8.3)	Tyr^2-2, 6	Tyr^2-C1, 3, 4, 5	
	4	155.9, C				
	NH		7.95, d (7.8)	Tyr^2-α, β	Tyr^2-Cα, β, Pro^2-CO	Ala^2-NH, Tyr^2-2, 6
Ala^2	CO	171.6, C				
	α	46.8, CH	4.59, p (7.2)	Ala^2-β, NH	Ala^2-CO, Cβ	Pro^1-δ
	β	17.1, CH$_3$	1.31,[b] d (6.8)	Ala^2-α, NH	Ala^2-CO, Cα	
	NH		7.74, d (7.4)	Ala^2-α, β	Ala^2-CO, Tyr^2-CO	Tyr^2-β, NH, Pro^1-α

[a]Sequential NOEs. [b]Overlapping signals.

instrument. Semipreparative reversed-phased HPLC (RP-HPLC) was performed using a Waters XBridge C18 column (Waters, 10 × 250 mm, 5 μm) connected to a Waters autopurification system consisting of a binary gradient module (Waters 2545), system fluidics organizer (Waters SFO), photodiode array detector (Waters 2998), mass spectrometer (Waters Acquity QDa), and sample manager (Waters 2767) with a Masslynx data handling system. Column chromatography was performed on ODS (15 μm, YMC Co.) and Sephadex LH-20 (18−110 μm, Pharmacia Co.). HPLC-grade CH_3CN and MeOH were purchased from Merck KGaA, and HPLC-grade H_2O was obtained by filtration using a Milli-Q Direct water purification system. Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories. 1-Fluoro-2,4-dinitrophenyl-5-L-/D-leucinamide (L-/D-FDLA) and all amino acid standards were purchased from Sigma-Aldrich Chemical Corporation.

Sponge Material. The marine sponge *Phakellia fusca* was collected off Yongxing Island in the South China Sea in May 2017 and was identified by Prof. Jin-He Li (Institute of Oceanology,

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Table 4. NMR Spectroscopic Data for Fuscasin D (4) at 600 MHz (^{1}H) and 150 MHz (^{13}C) in DMSO-d_6

residue	position	δ_C, type	δ_H, mult (J, Hz)	TOCSY	HMBC (H→C)	ROESY[a]
Pro^1	CO	171.07, C				
	α	60.5, CH	4.21, m	Pro^1-β, γ, δ	Pro^1-Cβ, γ, δ	Leu^1-α, NH
	β	31.2, CH_2	a: 2.33, dd (12.1, 6.3)	Pro^1-α, γ, δ	Pro^1-CO, Cδ	
			b: 2.06,[b] m	Pro^1-α, γ, δ	Pro^1-CO, Cα, δ	
	γ	22.0, CH_2	a: 1.84,[b] m	Pro^1-α, β, δ	Pro^1-Cα	
			b: 1.51,[b] m	Pro^1-α, β, δ		
	δ	46.7, CH_2	a: 3.39, m	Pro^1-α, β, γ		
			b: 3.31, m	Pro^1-α, β, γ	Pro^1-Cβ	
Leu^1	CO	170.2, C				
	α	49.7, CH	4.58, m	Leu^1-β, γ, δ, δ', NH		Pro^2-δ, Pro^1-α
	β	38.4, CH_2	a: 2.07[b], m	Leu^1-α, γ, δ, δ', NH	Leu^1-CO	
			b: 1.69, m	Leu^1-α, γ, δ, δ', NH	Leu^1-Cγ	
	γ	24.8, CH	1.72, m	Leu^1-α, β, δ, δ', NH		
	δ	21.3, CH_3	0.87, d (5.8)	Leu^1-α, β, γ, NH	Leu^1-Cβ, γ, δ'	
	δ'	23.1, CH_3	0.98, d (6.0)	Leu^1-α, β, γ, NH	Leu^1-Cβ, γ, δ	
	NH		9.03, d (8.1)	Leu^1-α, β, γ, δ, δ'	Pro^1-CO	Pro^2-α, Leu^2-α
Pro^2	CO	171.13, C				
	α	60.8, CH	4.04,[b] m	Pro^2-β, γ, δ		Leu^1-NH, Asp-α, NH
	β	29.1, CH_2	a: 2.06,[b] m	Pro^2-α, γ, δ	Pro^2-CO, Cα	
			b: 1.75,[b] m	Pro^2-α, γ, δ	Pro^2-Cγ,δ	
	γ	24.2, CH_2	a: 1.94, m	Pro^2-α, β, δ		
			b: 1.83,[b] m	Pro^2-α, β, δ	Pro^2-Cα	
	δ	47.5, CH_2	a: 4.02,[b] m	Pro^2-α, β, γ		Leu^1-α
			b: 3.65, m	Pro^2-α, β, γ		Leu^1-α
Asp	CO	171.6, C				
	α	50.4, CH	4.28, m	Asp-β, NH	Asp-CO	Pro^2-α
	β	36.4, CH_2	a: 2.56, m	Asp-α, NH	Pro^2-CO	
			b: 2.56, m	Asp-α, NH	Asp-CO, γ-CO	
	γ-CO	172.1				
	NH		8.05, brd	Asp-α, β		Pro^2-α
Tyr	CO	168.8, C				
	α	57.7, CH	4.18, m	Tyr-β	Tyr-CO, Cβ	Leu^2-α, NH
	β	36.9, CH_2	a: 2.81, m	Tyr-α	Tyr-CO, Cα, 1, 2, 6	
			b: 2.67, m	Tyr-α	Tyr-Cα, 1, 2, 6	
	1	127.1, C				
	2, 6	129.9, CH	6.98, d (8.1)	Tyr-3, 5	Tyr-Cβ, 2, 4, 6	
	3, 5	115.2, CH	6.65, d (8.0)	Tyr-2, 6	Tyr-Cβ, 1, 3−5	
	4	156.2, C				
	NH		7.67, brd	Tyr-α, β	Asp-CO	
Leu^2	CO	172.0, C				
	α	50.9, CH	4.35, dt (6.4, 5.2)	Leu^2-β, γ, δ, δ', NH	Leu^2-CO, Cβ, γ	Leu^3-NH, Tyr-α
	β	41.8, CH_2	a: 1.36, m	Leu^2-α, γ, δ, δ', NH	Leu^2-Cγ	
			b: 1.36, m	Leu^2-α, γ, δ, δ', NH	Leu^2-Cγ	
	γ	23.9, CH	1.37, m	Leu^2-α, β, δ, δ', NH	Leu^2-Cβ	
	δ	22.7, CH_3	0.82,[b] d (5.4)	Leu^2-α, β, γ, NH	Leu^2-Cβ, γ, δ'	
	δ'	22.8, CH_3	0.82[b], d (5.4)	Leu^2-α, β, γ, NH	Leu^2-Cβ, γ, δ	
	NH		7.17, d (7.0)	Leu^2-α, β, γ, δ, δ'	Tyr-CO	Tyr-α, Leu^3-NH
Leu^3	CO	170.4, C				
	α	51.4, CH	3.99, m	Leu^3-β, γ, δ, δ', NH	Leu^3-CO	Leu^1-NH
	β	38.3, CH_2	a: 1.52,[b] m	Leu^3-α, γ, δ, δ', NH	Leu^3-Cα	
			b: 1.29, m	Leu^3-α, γ, δ, δ', NH	Leu^3-CO	
	γ	24.1, CH	1.76,[b] m	Leu^3-α, β, δ, δ', NH		
	δ	20.7, CH_3	0.79, d (6.6)	Leu^3-α, β, γ, NH	Leu^3-Cβ, γ, δ'	
	δ'	23.4, CH_3	0.89, d (6.7)	Leu^3-α, β, γ, NH	Leu^3-Cβ, γ, δ	
	NH		8.56, brs	Leu^3-α, β, γ, δ, δ'	Leu^2-CO	Leu^2-α, Leu^2-NH

[a]Sequential NOEs. [b]Overlapping signals.

Chinese Academy of Sciences, People's Republic of China). A voucher sample (No. 2017051502) was deposited at the Marine Drugs Research Center, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, China.

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Extraction and Isolation. The sponge (4 kg, dry wt) was minced and exhaustively extracted with 95% EtOH, and combined EtOH extracts were evaporated to dryness under vacuum. The residue was then partitioned between H_2O and EtOAc, and the organic layer was further partitioned between 90% MeOH and petroleum ether. The remaining fraction after removal of the petroleum ether soluble extract (64.7 g) was concentrated under vacuum to yield 59.6 g and then was subjected to column chromatography on Sephadex LH-20 with CH_2Cl_2/MeOH (1:1, v/v) as eluent, affording four fractions (Fr.1−4). Fr.2 was fractionated using automated reversed-phase flash chromatography with a linear gradient from 10% aqueous MeOH to 100% MeOH over 300 min (flow rate 30.0 mL/min, UV detection at 200 nm) to afford 15 subfractions (Fr.2.A−O). Fr.2.G was separated by preparative reversed-phase HPLC (Waters XBridge C18, 5 μm, 19 × 250 mm, linear gradient, from 5% aqueous CH_3CN (0.1% formic acid) to 100% CH_3CN over 150 min, 20.0 mL/min) to yield 10 fractions. The fifth fraction was further purified by an automatic purification system guided by mass spectrometry using semi-preparative RP-HPLC (Waters XBridge C18, 5 μm, 10 × 250 mm, 5.0 mL/min) eluting with 25% aqueous CH_3CN (0.1% formic acid) to yield fuscasin B (**2**, 1.2 mg, t_R 15.9 min) and fuscasin C (**3**, 2.8 mg, t_R 17.3 min). Similarly, Fr.2.I.8 and Fr.2.J.3 were purified by the MS-guided isolation method mentioned above, eluting with 50−60% aqueous MeOH (0.1% formic acid) to yield fuscasin D (**4**, 3.7 mg, t_R 26.0 min) and fuscasin A (**1**, 9.1 mg, t_R 24.7 min), respectively.

Fuscasin A (1): yellowish, amorphous powder; $[\alpha]^{25}_D$ −97.8 (*c* 0.90, MeOH); IR (ATR) ν_{max} 3279, 3061, 2956, 2931, 2874, 1714, 1634, 1523, 1438, 1371, 1318, 1242, 1158, 1043, 1026, 922, 823, 751, 701 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; ESIMS/MS data, Figure 2; HRESIMS *m/z* 748.4034 $[M + H]^+$ (calcd for $C_{39}H_{54}N_7O_8$, 748.4034).

Fuscasin B (2): yellowish, amorphous powder; $[\alpha]^{25}_D$ −47.2 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 225 (3.85), 275 (3.59) nm; IR (ATR) ν_{max} 3586, 3289, 3183, 2957, 2926, 2852, 2363, 1634, 1575, 1556, 1539, 1515, 1446, 1158, 1373, 1346, 1260, 1240, 1170, 1084 cm^{-1}; ^1H and ^{13}C NMR data, Table 2; ESIMS/MS data, Figure S19; HRESIMS *m/z* 814.4574 $[M + H]^+$ (calcd for $C_{38}H_{60}N_{11}O_9$, 814.4575).

Fuscasin C (3): yellowish, amorphous powder; $[\alpha]^{25}_D$ −64.8 (*c* 0.25, MeOH); UV (MeOH) λ_{max} (log ε) 225 (4.06), 277 (3.45) nm; IR (ATR) ν_{max} 3302, 2926, 2872, 1626, 1514, 1450, 1372, 1346, 1237, 1186, 1169, 1099, 1043, 881, 827 cm^{-1}; ^1H and ^{13}C NMR data, Table 3; ESIMS/MS data, Figure S31; HRESIMS *m/z* 776.3983 $[M + H]^+$ (calcd for $C_{40}H_{54}N_7O_9$, 776.3983).

Fuscasin D (4): yellowish, amorphous powder; $[\alpha]^{25}_D$ −65.4 (*c* 0.32, MeOH); UV (MeOH) λ_{max} (log ε) 225 (3.90), 278 (3.24) nm; IR (ATR) ν_{max} 3280, 2954, 2921, 2852, 2322, 1631, 1515, 1442, 1389, 1366, 1230, 1202, 1174, 1096, 1044, 920, 800 cm^{-1}; ^1H and ^{13}C NMR data, Table 4; ESIMS/MS data, Figure S43; HRESIMS *m/z* 812.4567 $[M + H]^+$ (calcd for $C_{41}H_{62}N_7O_{10}$, 812.4558).

Absolute Configuration Assignments. Compounds **1**−**4** (0.1 mg each) were hydrolyzed with stirring in 6 N HCl (200 μL) at 110 °C for 12 h. The residual HCl fumes were removed under a N_2 stream. Acid hydrolysates (suspended in 50 μL of H_2O) were treated with 1 M $NaHCO_3$ (20 μL) and then with L-FDLA (100 μL of a 10 mg/mL solution in acetone), and the mixture was stirred at 37 °C for 1 h. The reaction was quenched with 1 N HCl (20 μL) and then diluted with MeOH for subsequent analysis. Authentic standards of L-Pro, L-Ala, L-Val, L-Phe, L-Leu, L-Tyr, L-Arg, and L-Asp were treated with L-FDLA and D-FDLA as described above and yielded the L-FDLA -L-amino acids and D-FDLA -L-amino acids standards. Marfey's derivatives of **1**−**4** were analyzed by UPLC-HRMS (Acquity UPLC HSS T3, 2.1 × 100 mm, 1.8 μm, 0.4 mL/min), and their retention times were compared with those from the authentic standard derivatives. Retention times for the derivatized amino acid standards are as follows: L-FDLA-L-Pro 14.33 min, D-FDLA-L-Pro 15.95 min; L-FDLA-L-Ala 14.19 min, D-FDLA-L-Ala 16.42 min; L-FDLA-L-Val 16.06 min, D-FDLA-L-Val 18.28 min; L-FDLA-L-Phe 17.24 min, D-FDLA-L-Phe 18.71 min; L-FDLA-L-Leu 17.15 min, D-FDLA-L-Leu 19.12 min; L-FDLA-L-Tyr (di) 20.04 min, D-FDLA-L-Tyr (di) 22.21 min; L-FDLA-L-Arg 10.03 min, D-FDLA-L-Arg 9.53 min; and L-FDLA-L-Asp 12.48 min, D-FDLA-L-Asp 13.19 min.

Cytotoxicity Assay. The cytotoxicity assay was performed according to a previous method.[13] The Cell Counting Kit-8 (CCK-8) method was used for *in vitro* evaluation of the cytotoxicities of compounds **1**−**4** against human cancer cell lines MCF-7, HeLa, NCI-H460, PC9, HepG2, and SW480 and nonmalignant cells (rat cardiomyoblast cell line H9C2). The MCF-7, HeLa, HepG2, and H9C2 cells were cultured at 37 °C in DMEM, while NCI-H460, PC9, and SW480 cell lines were grown in RPMI 1640. The medium was supplemented with 10% fetal bovine serum and antibiotics. The cell lines cited above (3×10^3 cells/well) were treated with test compounds for 72 h, and then 10 μL of CCK-8 solution was added. After 1 h of incubation at 37 °C (5% CO_2), the optical density (OD) was recorded at 450 nm by a microplate reader (SpectraMax 190, Molecular Devices). The half-maximal inhibitory concentration (IC_{50}) was calculated by fitting the data with a log (inhibitor) values response model of GraphPad Prism 5.0 software. Cisplatin was used as the positive control against cancer cell lines MCF-7, HeLa, NCI-H460, PC9, HepG2, and SW480, with IC_{50} values of 4.4, 4.8, 3.2, 2.9, 4.2, and 3.8 μM, respectively.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b01033.

1D and 2D NMR, HRESIMS, UV, IR spectra, and cytotoxicity data of **1**−**4**; HRESIMS/MS spectra and the advanced Marfey's analysis of **2**−**4** (PDF)

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Notes

The authors declare no competing financial interest.

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