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Antiplasmodial and antiviral cyclohexadepsipeptides from the endophytic fungus *Pullularia* sp. BCC 8613

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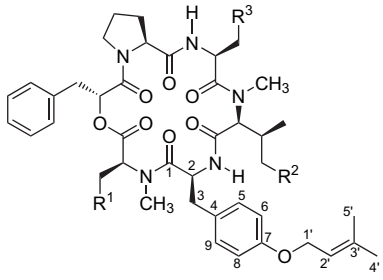
Abstract—Four new cyclohexadepsipeptides, pullularins A–D, were isolated from the endophytic fungus *Pullularia* sp. BCC 8613. Structures of these compounds were elucidated by interpretation of NMR spectroscopic and mass spectrometric data. The absolute configurations of amino acid and hydroxy acid residues were determined by HPLC analysis of depsipeptide acid hydrolyzates using a chiral column and Marfey's method. Pullularin A exhibited activities against the malarial parasite *Plasmodium falciparum* K1 (IC_{50} 3.6 μg/mL) and herpes simplex virus type 1 (HSV-1; IC_{50} 3.3 μg/mL), whereas it showed weak cytotoxicity to Vero cells (IC_{50} 36 μg/mL).

1. Introduction

The yeast-like fungus *Pullularia pullulans* (*Aureobasidium pullulans*) is well known as a producer of the exopolysaccharide (α-glucan), pullulan,[1] which has been produced in industrial scale by fermentation and utilized for various purposes.[2] Applications for the polymer are as films, oxygen-impermeable coatings, adhesives, and fibers.[3,4] However, to our knowledge there has been no reported isolation of low-molecular weight metabolites from the genus *Pullularia*. As a part of our research program focused on the identification of bioactive compounds from local fungi in Thailand,[5–7] we have investigated the constituents of an endophytic fungus *Pullularia* sp. BCC 8613 (isolated from a leaf of *Culophyllum* sp., collected in Hala–Bala Wildlife Sanctuary, Narathiwat Province), whose culture extract had shown modest cytotoxic activity to cancer cell lines. This study led to the isolation of four new cyclohexadepsipeptides, pullularins A–D (**1**–**4**), possessing an unusual *O*-prenyl-L-tyrosine residue. We report herein the isolation, structure elucidation, and biological activities of these compounds.



1: R^1 = H, R^2 = CH_3, R^3 = OH
2: R^1 = CH_3, R^2 = CH_3, R^3 = OH
3: R^1 = H, R^2 = H, R^3 = OH
4: R^1 = H, R^2 = CH_3, R^3 = H

2. Results and discussion

Pullularin A (**1**) was isolated as the most abundant cyclic depsipeptide constituent of the EtOAc extract from *Pullularia* sp. BCC 8613 fermentation broth. The molecular formula of **1** was determined by HRMS (ESI-TOF) and ^{13}C NMR as $C_{42}H_{57}N_5O_9$. The IR spectrum showed a sharp absorption band of an ester at ν_{max} 1747 cm^{-1} and intense broad absorption bands of amides at 1640 cm^{-1}. The ^1H and ^{13}C NMR spectroscopic data suggested that **1** was a hexadepsipeptide, showing six carbonyl carbon resonances at δ_C 172.6, 171.5, 169.7, 168.5, 167.9, and 166.3, and six α-protons at δ_H 5.67 (dd, *J*=9.4, 4.5 Hz), 4.84 (m), 4.77 (dd, *J*=7.2, 3.5 Hz), 4.70 (d, *J*=10.8 Hz), 4.53 (dd, *J*=8.6, 1.8 Hz), and 3.70 (q, *J*=6.7 Hz). Two amide protons (N*H*) at δ_H 9.56 (d, *J*=8.8 Hz) and 8.37 (br s), and two signals of *N*-methylamide protons at δ_H 3.04 (3H, s) and 2.53 (3H, s) were also observed. One of the residues was assigned to be 3-phenyllactic acid (3-Ph-Lac) on the basis of the 2D NMR data

Keywords: *Pullularia* sp.; Antiplasmodial activity; Antiviral activity; Cyclodepsipeptides.
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(COSY, HMQC, and HMBC). An oxymethine at δ_C 71.9 (C-2; δ_H 5.67 (dd, J=9.4, 4.5 Hz)) was attached with methylene at δ_C 36.1 (C-3; δ_H 3.25 (m) and 2.94 (dd, J=12.8, 4.5 Hz)), which was flanked by an unsubstituted phenyl group. Five amino acid residues were also elucidated based on their 2D COSY, HMQC, and HMBC data; an *N*-methylalanine (*N*-Me-Ala), an *O*-prenyltyrosine (*O*-prenyl-Tyr), an *N*-methylisoleucine (*N*-Me-Ile), a serine (Ser), and a proline (Pro). Local structure of the unusual *O*-prenyl-Tyr was deduced from HMBC correlation from the methylene protons of the prenyl group (δ_H 4.49 (2H, d, J=6.5 Hz, H-1′)) to δ_C 157.4 quaternary carbon (C-7) of Tyr, and intense NOESY crosspeak between H-1′ and H-6/H-8 (δ_H 6.88 (d, J=8.5 Hz)). The sequence of the six residues was determined by HMBC and NOESY data (Fig. 1). Thus, α-protons of 3-Ph-Lac, *N*-Me-Ala, *O*-prenyl-Tyr, and *N*-Me-Ile were correlated, respectively, to carbonyl carbons of *N*-Me-Ala, *O*-prenyl-Tyr, *N*-Me-Ile, and Ser. The amide N*H* of Ser showed HMBC correlation to the carbonyl carbon (C-1) of Pro. The Pro-Ser connection was also supported by the NOESY correlations between the N*H* of Ser and β-methylene protons (H-3; δ_H 1.68, 1.47) of Pro. Although HMBC correlations from H-2 or H-5 of Pro to C-1 of 3-Ph-Lac were not observed, the 3-Ph-Lac-Pro linkage was indicated by the NOESY crosspeaks between H-2 (δ_H 5.67) of 3-Ph-Lac and H-5 methylene protons (δ_H 3.95 and 3.28) of Pro.

The IR and UV–vis spectra of pullularin B (**2**) were very similar to those of **1**. The molecular formula of **2** was determined by HR(ESI)MS and the ^{13}C NMR spectrum as $C_{43}H_{59}N_5O_9$. The ^{1}H and ^{13}C NMR spectra of **2** were closely related to those of **1** except for the replacement of the *N*-Me-Ala residue to 2-methylaminobutyric acid (*N*-Me-Abu). Instead of the doublet signal of the methyl group (δ_H 1.21; δ_C 13.6, C-3) in **1**, a triplet signal of methyl protons at δ_H 0.59 (δ_C 10.0, C-4) and resonances of diastereotopic methylene protons at δ_H 1.82 and 1.68 (δ_C 21.6, C-3) were present in **2**. Therefore, **2** contains a 2-methylaminobutyric acid (*N*-Me-Abu) residue, instead of *N*-Me-Ala. Interpretation of the 2D NMR spectroscopic data confirmed that the other four amino acid residues and 3-Ph-Lac were identical to those of **1**.

The HRMS and ^{13}C NMR spectroscopic data of pullularin C (**3**) indicated its molecular formula to be $C_{43}H_{59}N_5O_9$, which is lacking one CH_2 from **1**. Resonances of the methylene protons assigned to *N*-Me-Ile of **1** (δ_H 1.42 and 1.08; δ_C 25.7) were absent in pullularin C (**3**). The presence of *N*-methylvaline (*N*-Me-Val) residue, in the place of *N*-Me-Ile, was confirmed by analysis of COSY and HMQC spectroscopic data. The δ_H 4.62 (d, J=10.8 Hz, H-2; δ_C 65.8, C-2) methine proton showed COSY crosspeak with the δ_H 2.42 methine (H-3; δ_C 26.0), which was further attached with two methyl groups, δ_H 1.03 (3H, d, J=6.4 Hz; δ_C 19.2) and 0.93 (3H, d, J=6.9 Hz; δ_C 18.2). The assignment of the *N*-methyl group of this residue (δ_H 2.56, 3H, s; δ_C 28.1) was revealed by the HMBC correlations from the *N*-methyl protons to C-1 (δ_C 65.8) and from H-2 (δ_H 4.62) to the *N*-methyl carbon. The carbonyl carbon of this residue (δ_C 168.0, C-1) was correlated from H-1 of the same residue and N*H* of *O*-prenyl-Tyr. On the other hand, H-1 and *N*-methyl protons of this residue (*N*-Me-Val) showed HMBC correlation to C-1 (δ_C 171.7) of Ser. These data indicated a partial sequence of Ser–*N*-Me-Val–*O*-prenyl-Tyr. The other sequences of the six residues were established in a similar manner as described for **1**.

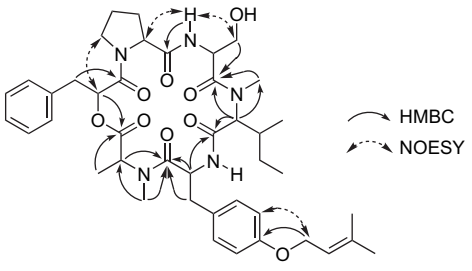
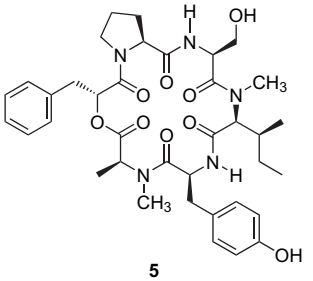


Figure 1. Selected HMBC and NOESY correlations for **1**.

Pullularin D (**4**) was obtained in very low quantity (1.2 mg). The HRMS experiment revealed the molecular formula of **4** as $C_{42}H_{57}N_5O_8$, therefore, lacking one oxygen atom from **1**. The IR spectrum of **4** was very similar to those of other pullularins. Analysis of NMR spectroscopic data revealed that the Ser residue of **1** is replaced by alanine (Ala) in pullularin D (**4**).

Attempts to crystallize **1** for X-ray diffraction analysis were unsuccessful. The most abundant metabolite (**1**) was treated with *p*-TsOH–Pd/C in H_2O/MeOH (50 °C, 3 days) to obtain the deprenyl analog **5** in 69% yield.[8,9] Pullularin A (**1**) was also converted to its 3,5-dinitrobenzoate derivative by acylation of the serine hydroxyl group. Unfortunately, these derivatives did not give crystals suitable for X-ray crystallography.



5

The absolute configurations of 3-Ph-Lac and amino acid residues were addressed by combination of chiral column HPLC analysis of the acid hydrolyzates[10] and Marfey's method.[11,12] Pullularins A (**1**) and C (**3**) were hydrolyzed in 6 M HCl (110 °C, 15 h) and the hydrolyzates were subjected to HPLC analysis using a ligand-exchange-type chiral column (see Section 3). Co-injection with standard L- and D-3-Ph-Lac and L- and D-amino acids confirmed that both hydrolyzates contained (2*R*)-D-3-Ph-Lac, *O*-prenyl-L-Tyr, and L-Pro. The 2*S* configuration was addressed for *N*-Me-Ile in **1** and *N*-Me-Val in **3**, however, in the former case, (2*S*,3*S*)-*N*-Me-Ile and (2*S*,3*R*)-*N*-Me-*allo*-Ile could not be distinguished. Also, peak separations of *N*-Me-L-Ala/*N*-Me-D-Ala and L-Ser/D-Ser using the chiral column met with failure, therefore, Marfey's method was employed. Hydrolyzate of pullularin A (**1**) was derivatized with N_α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA). HPLC analysis using an ODS column revealed that the hydrolyzate contained

L-Ser, *N*-Me-L-Ala, and (2*S*,3*S*)-*N*-Me-L-Ile. The defined absolute structure of pullularin A (**1**) is *cyclo*-[D-3-Ph-Lac–L-Pro–L-Ser–*N*-Me-L-Ile–*O*-prenyl-L-Tyr–*N*-Me-L-Ala]. Based on the close ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2) and the co-occurrence from the fungal strain BCC 8613, we propose that pullularins B (**2**), C (**3**), and D (**4**) should possess the same sense of absolute configurations as those of **1**.

Pullularins A (**1**), B (**2**), and C (**3**) and compound **5** were subjected to several biological assays: antimalarial (*Plasmodium falciparum* K1),[13] antitubercular (*Mycobacterium tuberculosis* $H_{37}Ra$),[14] antiviral (herpes simplex virus type 1; HSV-1)[15] activities, and cytotoxicity[15] to three cancer cell lines (KB, BC, and NCI-H187) and noncancerous Vero cells (Table 3). Because of the sample shortage, pullularin D (**4**) was not tested. Pullularins A (**1**) exhibited antimalarial activity (IC_{50} 3.6 μg/mL) and anti-HSV-1 activity (IC_{50} 3.3 μg/mL), as well as moderate antitubercular activity (MIC 25 μg/mL). This compound did not inhibit the growth of three cancer cell lines up to 20 μg/mL, and it showed weak cytotoxicity to Vero cells (IC_{50} 36 μg/mL). Pullularins B (**2**) and C (**3**) exhibited weaker activities in these assays when compared with **1**. It should be noted that compound **5**, the deprenyl analog of pullularin A, was inactive in these assays. This may due to the low lipophilicity of **5** compared to that of **1**. Further works on the optimization of fermentation conditions for pullularin A mass production, and SAR studies based on the replacement of the prenyl group of **1** with other alkyl groups are in progress.

Table 1. ^{13}C NMR spectroscopic data for pullularins A–D (acetone-d_6, 125 MHz)

Position	1	2	3	4
3-Ph-Lac				
1 (*C*=O)	166.3 (s)	165.8 (s)	165.8 (s)	165.7 (s)
2	71.9 (d)	71.7 (d)	72.0 (d)	72.0 (d)
3	36.1 (t)	36.4 (d)	36.2 (t)	36.1 (t)
4	136.6 (s)	136.9 (s)	136.8 (s)	136.8 (s)
5,9	129.7 (d)	129.8 (d)	129.8 (d)	129.8 (d)
6,8	128.3 (d)	128.2 (d)	128.3 (d)	128.2 (d)
7	126.5 (d)	126.4 (d)	126.5 (d)	126.4 (d)
Pro				
1 (*C*=O)	172.6 (s)	172.9 (s)	172.9 (s)	172.2 (s)
2	59.1 (d)[a]	58.9 (d)	59.2 (d)	58.8 (d)
3	29.3 (t)	29.4 (t)	29.4 (t)	29.2 (t)
4	24.1 (t)	24.2 (t)	24.1 (t)	24.0 (t)
5	46.3 (t)	46.2 (t)	46.1 (t)	46.0 (t)
Ser/Ala				
1 (*C*=O)	171.5 (s)	171.8 (s)	171.7 (s)	173.8 (s)
2	51.8 (d)	51.8 (d)	51.7 (d)	44.9 (d)
3	62.2 (t)	62.1 (t)	62.1 (t)	16.9 (q)
N-Me-Ile/*N*-Me-Val				
1 (*C*=O)	167.9 (s)	168.1 (s)	168.0 (s)	168.2 (s)
2	65.4 (d)	65.4 (d)	65.8 (d)	65.4 (d)
3	32.5 (d)	32.6 (d)	26.0 (d)	32.5 (d)
4	24.8 (t)	24.8 (t)	19.2 (q)	24.9 (t)[a]
5	11.4 (q)	11.4 (q)	—	11.3 (q)
3-*C*H_3	15.7 (q)	15.8 (q)	18.2 (q)	15.6 (q)
N-*C*H_3	28.1 (q)	28.3 (q)	28.1 (q)	28.2 (q)
O-prenyl-Tyr				
1 (*C*=O)	168.5 (s)	168.3 (s)	168.5 (s)	168.5 (s)
2	52.2 (d)	52.6 (d)	52.0 (d)	52.2 (d)
3	37.3 (t)	37.1 (t)	37.3 (t)	37.3 (t)
4	130.2 (s)	130.3 (s)	130.3 (s)	130.5 (s)
5,9	130.4 (d)	130.4 (d)	130.4 (d)	130.4 (d)
6,8	114.1 (d)	114.2 (d)	114.2 (d)	114.2 (d)
7	157.4 (s)	157.5 (s)	157.5 (s)	157.5 (s)
1′	64.4 (t)	64.4 (t)	64.4 (t)	64.4 (t)
2′	120.4 (d)	120.5 (d)	120.5 (d)	120.5 (d)
3′	136.4 (s)	136.4 (s)	136.5 (s)	136.4 (s)
4′	24.8 (q)	24.9 (q)	24.9 (q)	24.9 (q)[a]
5′	17.2 (q)	17.3 (q)	17.3 (q)	17.3 (q)
N-Me-Ala/*N*-Me-Abu				
1 (*C*=O)	169.7 (s)	169.3 (s)	169.7 (s)	169.7 (s)
2	59.1 (d)[a]	65.3 (d)	58.9 (d)	59.2 (d)
3	12.7 (q)	21.6 (t)	12.7 (q)	12.7 (q)
4	—	10.0 (q)	—	—
N-*C*H_3	35.8 (q)	37.6 (q)	35.8 (q)	35.8 (q)

[a] The ^{13}C signals are overlapped.

3. Experimental

3.1. General procedures

Melting points were measured with an Electrothermal IA9100 digital melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO P-1030 digital polarimeter. UV spectra were recorded on a Varian CARY 1E UV–vis spectrophotometer. FTIR spectra were taken on a Bruker VECTOR 22 spectrometer. NMR spectra were taken on Bruker DRX400 and AV500D spectrometers. ESI-TOF mass spectra were measured with a Micromass LCT mass spectrometer.

3.2. Fungal material

Pullularia sp. was isolated from a leaf of *Culophyllum* sp. (Guttiferae) collected in Hala–Bala Wildlife Sanctuary, Narathiwat Province, Thailand, by one of the authors (T.S.), and it was identified by Prof. E. B. G. Jones, BIOTEC. This fungus was deposited in the BIOTEC Culture Collection (BCC) as BCC 8613 on April 22, 2000.

3.3. Fermentation and isolation

Pullularia sp. BCC 8613 was fermented in potato dextrose broth medium (20×250 mL) under static conditions at 25 °C for 19 days. The cultures were harvested by filtration. The filtrate was extracted twice with EtOAc (5 L) and the combined organic layer was concentrated under reduced pressure to leave a brown gum (705 mg). This crude extract was passed through a Sephadex LH-20 column (4.5×25 cm) with MeOH as eluant. The 160–240 mL elute contained cyclodepsipeptides (217 mg). After repeating this process twice, the cyclodepsipeptide-containing fraction (146 mg) was subjected to preparative HPLC using a reversed phase column (NovaPak HR C_{18}, 2.5×10.0 cm; MeOH/H_2O=70:30; flow rate, 8 mL/min) to obtain **3** (13.3 mg, t_R 30 min), **1** (59.4 mg, t_R 36 min), and a mixture of **1**, **2** and **4** (15.3 mg, 40–55 min). The last fraction was further separated by preparative HPLC using MeCN/H_2O=50:50 as eluant to afford **1** (6.5 mg, t_R 24 min), **2** (4.9 mg, t_R 34 min), and **4** (1.2 mg, t_R 41 min).

3.3.1. Pullularin A (1). Colorless solid; mp 128–130 °C; $[\alpha]_D^{23}$ −140 (*c* 0.42, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.67), 225 (sh) (4.30), 279 (3.40) nm; IR (KBr) ν_{max} 3462, 3264, 1747, 1651 (br), 1509, 1457 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRMS (ESI-TOF) *m/z* 798.4044 $[M+Na]^+$ (calcd for $C_{42}H_{57}N_5O_9Na$, 798.4054).

Table 2. ^{1}H NMR spectroscopic data for pullularins A–D (acetone-d_6, 500 MHz)

Position	**1**	**2**	**3**	**4**
3-Ph-Lac				
2	5.67 (dd, 9.4, 4.5)	5.70 (dd, 8.6, 5.4)	5.63 (dd, 9.2, 4.6)	5.62 (dd, 9.2, 4.7)
3	3.25 (m)	3.23 (dd, 13.0, 8.6)	3.26 (dd, 12.6, 9.3)	3.26 (dd, 12.8, 9.5)
	2.94 (dd, 12.8, 4.5)	2.89 (dd, 13.0, 5.4)	2.89 (dd, 12.8, 4.5)	2.87 (dd, 12.8, 4.6)
5,9	7.26–7.28 (2H, m)	7.25–7.27 (2H, m)	7.25–7.27 (2H, m)	7.25–7.27 (2H, m)
6,8	7.26–7.28 (2H, m)	7.25–7.27 (2H, m)	7.25–7.27 (2H, m)	7.25–7.27 (2H, m)
7	7.19 (m)	7.18 (m)	7.19 (m)	7.19 (m)
Pro				
2	4.53 (dd, 8.6, 1.8)	4.53 (dd, 8.7, 2.0)	4.53 (m)	4.40 (dd, 8.6, 2.1)
3	2.07 (m)	2.08 (m)	2.04–2.08 (2H, m)	2.04–2.07 (2H, m)
	1.87–1.98 (m)	1.85–1.90 (m)		
4	1.87–1.98 (2H, m)	1.85–1.90 (2H, m)	1.93 (m)	1.89 (m)
			1.87 (m)	1.84 (m)
5	3.95 (m)	3.90 (m)	3.95 (m)	3.96 (dt, 3.5, 9.0)
	3.28 (m)	3.26 (m)	3.24 (m)	3.23 (m)
Ser/Ala				
2	4.77 (dd, 7.2, 3.5)	4.68 (m)	4.73 (m)	4.72 (m)
3	3.83 (dd, 11.8, 7.4)	3.77–3.79 (2H, m)	3.77–3.79 (2H, m)	1.32 (3H, d, 6.7)
	3.76 (dd, 11.8, 3.5)			
N*H*	8.37 (br s)	8.28 (d, 3.4)	8.20 (br s)	8.09 (d, 4.7)
N-Me-Ile/*N*-Me-Val				
2	4.70 (d, 10.8)	4.69 (d, 10.7)	4.62 (d, 10.8)	4.65 (d, 10.9)
3	2.13 (m)	2.17 (m)	2.42 (m)	2.14 (m)
4	1.42 (m)	1.41 (m)	1.03 (3H, d, 6.9)	1.38 (m)
	1.08 (m)	1.09 (m)		1.07 (m)
5	0.97 (3H, t, 7.2)	0.97 (3H, t, 7.3)	—	0.97 (3H, t, 7.4)
3-CH_3	0.98 (3H, d, 6.2)	0.99 (3H, d, 6.5)	0.93 (3H, d, 6.9)	1.00 (3H, d, 6.4)
N-CH_3	2.53 (3H, s)	2.59 (3H, s)	2.56 (3H, s)	2.56 (3H, s)
O-prenyl-Tyr				
2	4.84 (m)	4.82 (m)	4.82 (dt, 4.2, 9.0)	4.78 (dt, 3.9, 9.0)
3	3.11 (dd, 13.3, 9.2)	3.04 (m)	3.09 (dd, 13.2, 9.1)	3.08 (dd, 13.1, 9.6)
	2.55 (dd, 13.3, 4.6)	2.58 (m)	2.52 (dd, 13.2, 4.3)	2.52 (dd, 13.1, 3.9)
5,9	7.13 (2H, d, 8.7)	7.13 (2H, d, 8.7)	7.12 (2H, d, 8.6)	7.13 (2H, d, 8.6)
6,8	6.78 (2H, d, 8.5)	6.78 (2H, d, 8.6)	6.78 (2H, d, 8.7)	6.78 (2H, d, 8.7)
1′	4.49 (2H, d, 6.5)	4.49 (2H, d, 6.5)	4.48 (2H, d, 6.2)	4.48 (2H, d, 6.5)
2′	5.43 (m)	5.42 (m)	5.42 (m)	5.42 (m)
4′	1.75 (3H, s)	1.75 (3H, s)	1.74 (3H, s)	1.73 (3H, s)
5′	1.72 (3H, s)	1.73 (3H, s)	1.71 (3H, s)	1.71 (3H, s)
N*H*	9.56 (d, 8.8)	9.53 (d, 8.5)	9.53 (d, 8.8)	9.58 (d, 8.6)
N-Me-Ala/*N*-Me-Abu				
2	3.70 (q, 6.7)	3.45 (dd, 8.4, 5.7)	3.68 (q, 6.5)	3.68 (q, 6.7)
3	1.21 (3H, d, 6.7)	1.82 (m)	1.19 (3H, d, 6.7)	1.18 (3H, d, 6.7)
		1.68 (m)		
4	—	0.59 (3H, t, 7.5)	—	—
N-CH_3	3.04 (3H, s)	3.08 (3H, s)	3.03 (3H, s)	3.03 (3H, s)

3.3.2. Pullularin B (2). Colorless solid; mp 122–124 °C; $[\alpha]_D^{23}$ −116 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.65), 225 (sh) (4.37), 278 (3.44) nm; IR (KBr) ν_{max} 3447, 1748, 1648 (br), 1509, 1457 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRMS (ESI-TOF) *m*/*z* 812.4196 $[M+Na]^+$ (calcd for $C_{43}H_{59}N_5O_9Na$, 812.4210).

3.3.3. Pullularin C (3). Colorless solid; mp 112–113 °C; $[\alpha]_D^{24}$ −137 (*c* 0.21, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.55), 225 (sh) (4.29), 281 (3.49) nm; IR (KBr) ν_{max} 3438 (br), 3271, 1746, 1645 (br), 1512, 1455, 756 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRMS (ESI-TOF) *m*/*z* 784.3906 $[M+Na]^+$ (calcd for $C_{41}H_{55}N_5O_9Na$, 784.3897).

Table 3. Biological activities of the cyclodepsipeptides **1**–**3** and **5**

Compound	Antimalaria[a] (IC_{50}, μg/mL)	Antituberculosis[b] (MIC, μg/mL)	Anti-HSV-1 (IC_{50}, μg/mL)	Cytotoxicity (IC_{50}, μg/mL)[c]			
				KB	BC	NCI-H187	Vero
Pullularin A (**1**)	3.6	25	3.3	>20	>20	>20	36
Pullularin B (**2**)	3.3	100	49	>20	>20	>20	>50
Pullularin C (**3**)	9.8	50	>50	>20	>20	>20	>50
Compound **5**	>20	200	—[f]	—[f]	—[f]	—[f]	>50
Dihydroartemisinin[d]	0.0011	—[f]	—[f]	—[f]	—[f]	—[f]	—[f]
Acyclovir[e]	—[f]	—[f]	1.9	—[f]	—[f]	—[f]	>50

[a] Inhibition of the proliferation of *Plasmodium falciparum* K1.
[b] Growth inhibitory activity against *Mycobacterium tuberculosis* $H_{37}Ra$. The MIC value for a standard antituberculosis drug, isoniazid, was 0.050 μg/mL.
[c] The IC_{50} values of a standard compound, ellipticine, against KB, BC, NCI-H187, and Vero cells were 0.27, 0.21, 0.15, and 0.60 μg/mL, respectively.
[d] Standard antimalarial compound.
[e] Standard compound for the anti-HSV-1 assay.
[f] Not tested.

3.3.4. Pullularin D (4). Colorless solid; IR (KBr) ν_{max} 3443, 3261, 1745, 1655 (br), 1510, 1453 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRMS (ESI-TOF) *m/z* 782.4108 $[M+Na]^+$ (calcd for $C_{42}H_{57}N_5O_8Na$, 782.4105).

3.4. Deprenylation of 1

To a solution of **1** (15.2 mg) in MeOH (0.5 mL) and H_2O (0.1 mL) were added 10% Pd/C (10 mg) and *p*-TsOH·H_2O (5 mg). The resulting suspension was stirred at 50 °C for 3 days, then K_2CO_3 (s) (10 mg) was added. The mixture was diluted with EtOAc (2 mL) and filtered. The filtrate was evaporated, and the residue was redissolved in EtOAc, washed with aq. NH_4Cl, and H_2O. The organic layer was evaporated to leave a light brown solid. This crude product was purified by preparative HPLC (NovaPak HR C_{18}, 2.5×10.0 cm; MeOH/H_2O=60:40; flow rate, 8 mL/min) to afford compound **5** (t_R 15 min, 9.6 mg, 69%) as a colorless solid.

3.4.1. Deprenylpullularin A (5). Colorless amorphous solid; $[\alpha]_D^{28}$ −78 (*c* 0.16, MeOH); ^1H NMR (500 MHz, $CDCl_3$) δ D-3-Ph-Lac: 7.28 (2H, m, H-5 and H-9), 7.19 (1H, m, H-7), 7.18 (2H, m, H-6 and H-8), 5.62 (1H, dd, *J*=9.2, 4.5 Hz, H-2), 3.26 (1H, m, H-3a), 3.23 (1H, m, H-3b); L-Pro: 4.08 (1H, m, H-2), 3.86 (1H, m, H-5a), 3.12 (1H, m, H-5b), 2.01 (1H, m, H-4a), 1.82 (2H, m, H-3a and H-3b), 1.75 (1H, m, H-4b); L-Ser: 8.54 (1H, br s, N*H*), 4.89 (1H, m, H-2), 3.97 (1H, m, H-3a), 3.61 (1H, br d, *J*=12.5 Hz, H-3b); *N*-Me-L-Ile: 4.53 (1H, d, *J*=9.9 Hz, H-2), 2.11 (3H, s, N-CH_3), 2.01 (1H, m, H-3), 1.25 (1H, m, H-4a), 0.87 (3H, m, H-5), 0.85 (3H, m, 3-CH_3), 0.83 (1H, m, H-4b); L-Tyr: 9.49 (1H, d, *J*=8.9 Hz, N*H*), 6.97 (2H, d, *J*=8.0 Hz, H-5 and H-9), 6.72 (2H, d, *J*=8.0 Hz, H-6 and H-8), 5.00 (1H, dt, *J*=6.4, 8.7 Hz, H-2), 3.06 (1H, m, H-3a), 2.63 (1H, dd, *J*=13.5, 9.0 Hz, H-3b); *N*-Me-L-Ala: 3.52 (1H, q, *J*=6.6 Hz, H-2), 2.98 (3H, s, N-CH_3), 1.41 (3H, d, *J*=6.6 Hz, H-3); ^{13}C NMR (125 MHz, $CDCl_3$) δ D-3-Ph-Lac: 168.5 (s, C-1), 135.3 (s, C-4), 129.7 (d, C-5 and C-9), 128.6 (d, C-6 and C-8), 127.0 (d, C-7), 72.7 (d, C-2), 36.5 (t, C-3); L-Pro: 172.3 (s, C-1), 60.1 (d, C-2), 47.4 (t, C-5), 29.0 (t, C-3), 24.6 (t, C-4); L-Ser: 171.6 (s, C-1), 63.4 (t, C-3), 53.5 (d, C-2); *N*-Me-L-Ile: 167.8 (s, C-1), 65.7 (d, C-2), 32.2 (d, C-3), 28.4 (q, N-CH_3), 25.3 (t, C-4), 16.2 (q, 3-CH_3), 12.2 (q, C-5); L-Tyr: 169.6 (s, C-1), 155.7 (s, C-7), 130.6 (d, C-5 and C-9), 128.2 (s, C-4), 115.4 (d, C-6 and C-8), 37.2 (t, C-3); *N*-Me-L-Ala: 170.0 (s, C-1), 59.4 (d, C-2), 36.2 (q, N-CH_3), 13.4 (q, C-3); HRMS (ESI-TOF) *m/z* 708.3619 $[M+H]^+$ (calcd for $C_{37}H_{50}N_5O_9$, 708.3608).

3.5. Determination of the absolute configuration

3.5.1. Acid hydrolysis of 1 and 3. Compound **1** (1.0 mg) was hydrolyzed with 6 M HCl (1 mL) at 110 °C for 15 h. After concentration to dryness, the residue was dissolved in MeOH (100 μL) and subjected to HPLC analysis. Hydrolysis of **3** was also conducted in the same manner.

3.5.2. Preparation of standard amino acids *O*-prenyl-L-Tyr and *O*-prenyl-D-Tyr. *N*-Boc-L-Tyr (Fluka) was treated with prenyl bromide and K_2CO_3 (s) in 2-butanone. Usual aqueous workup and purification by silica gel column chromatography gave *N*-Boc-*O*-prenyl-L-Tyr prenyl ester. Alkaline hydrolysis (2 M NaOH/MeOH) of this compound afforded *N*-Boc-*O*-prenyl-L-Tyr, which was purified by silica gel column chromatography. A portion (1 mg) was dissolved in trifluoroacetic acid (TFA, 0.4 mL), and the solution was stirred at rt for 0.5 h, then evaporated to dryness. The residue was dissolved in MeOH (150 μL), and used for HPLC analysis as authentic standard *O*-prenyl-L-Tyr. Similarly, *O*-prenyl-D-Tyr standard was synthesized in three steps from *N*-Boc-D-Tyr methyl ester (Fluka).

3.5.3. HPLC analysis of the depsipeptide hydrolyzates using a chiral column. HPLC analysis of the depsipeptide hydrolyzates was performed using a ligand-exchange-type chiral column: SUMICHIRAL OA-5000, 5 μm, 4.6× 150 mm (Sumika Chemical Analysis Service, Ltd); flow rate 1 mL/min, UV 235 nm.[10] Standard L- and D-amino acids and 3-phenyllactic acid were used for co-injection experiments. Two mobile phase conditions were employed for polarity reasons: (1) 15% isopropanol in 2 mM aq. $CuSO_4$, L-3-Ph-Lac (t_R 62 min), D-3-Ph-Lac (t_R 73 min), *O*-prenyl-L-Tyr (t_R 86 min), *O*-prenyl-D-Tyr (t_R 97 min); (2) 10% MeOH in 2 mM aq. $CuSO_4$, L-Pro (t_R 4.8 min), D-Pro (t_R 8.7 min), *N*-Me-L-Val (t_R 6.6 min), *N*-Me-D-Val (t_R 7.3 min). The hydrolyzate of **1** contained (2*R*)-D-3-Ph-Lac, *O*-prenyl-L-Tyr, and L-Pro. Peak separations of *N*-Me-L-Ala/*N*-Me-D-Ala and L-Ser/D-Ser were unsuccessful under various solvent system, also, peaks of (2*S*,3*S*)-*N*-Me-L-Ile and (2*S*,3*R*)-*N*-Me-L-*allo*-Ile overlapped. The hydrolyzate of **3** contained peaks of (2*R*)-D-3-Ph-Lac, *O*-prenyl-L-Tyr, L-Pro, and *N*-Me-L-Val. In both hydrolyzates, L-Tyr was detected, indicating partial deprenylation of *O*-prenyl-L-Tyr during acid hydrolysis.

3.5.4. Preparation and HPLC analysis of Marfey derivatives. Pullularin A (**1**, 10.0 mg) was hydrolyzed (6 M HCl), dried in vacuo. The hydrolyzate were added H_2O (1.5 mL), FDAA (Marfey's reagent, 30 mg) in acetone (3 mL), and 1 M $NaHCO_3$ (0.5 mL). The mixture was heated on a 40 °C bath for 3 h, quenched by addition of 1 M HCl (0.55 mL). The reaction mixture was partially concentrated using a rotary evaporator, the residue was extracted with EtOAc and concentrated under reduced pressure to furnish a yellow solid (42 mg). A small portion of this product (0.2 mg) was dissolved in MeOH and subjected to HPLC analysis using an ODS column (NovaPak HR C_{18}, 3.9×150 mm, 4 μm; flow rate 0.5 mL/min). Retention times of the standard FDAA derivatives of L- and D-Ser were 14.6 and 15.4 min, respectively, using the mobile phase of MeCN/(0.05% TFA in H_2O)=20:80. Retention times of the standard FDAA derivatives of (2*S*,3*S*)-*N*-Me-L-Ile and (2*S*,3*R*)-*N*-Me-L-*allo*-Ile were 21.7 and 22.6 min, respectively, using the mobile phase of MeCN/(0.05% TFA in H_2O)=35:65. The chromatograms of the FDAA-derivatized hydrolyzate showed peaks of L-Ser and *N*-Me-L-Ile, and these assignments were confirmed by co-injections. However, peaks of *N*-Me-L-Ala and *N*-Me-D-Ala overlapped under varied proportion of the solvents. This last problem was solved by replacement of 0.05% TFA with 20 mM ammonium phosphate (pH 4.6). With the solvent system of MeCN/(20 mM ammonium phosphate in H_2O)=15:85, retention times of *N*-Me-L-Ala and *N*-Me-D-Ala were 26.0 and 34.7 min, respectively. Under the same conditions the derivatized hydrolyzate showed peaks of L-Ser (8.0 min), L-Pro (16.2 min), and *N*-Me-L-Ala (26.1 min), indicating the L-configuration of *N*-Me-Ala in **1**.

3.6. Biological assays

Assay for activity against *P. falciparum* (K1, multi-drug resistant strain) was performed using the microculture radioisotope technique described by Desjardins et al.[13] Growth inhibitory activity against *M. tuberculosis* $H_{37}Ra$ was performed using the Microplate Alamar Blue Assay (MABA) described by Collins and Franzblau.[14] Anti-HSV-1 activity and cytotoxicity against KB cells (oral human epidermoid carcinoma), BC cells (human breast cancer), NCI-H187 cells (human small cell lung cancer), and Vero cells (African green monkey kidney fibroblast) were evaluated using the colorimetric method.[15] Vero cells were also used as host cells in the anti-HSV-1 assay.

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References and notes

1. Bender, H.; Lehmann, J.; Wallenfels, K. *Biochim. Biophys. Acta* **1959**, *36*, 309–316.
2. Shingel, K. I. *Carbohydr. Res.* **2004**, *339*, 447–460.
3. Yuen, S. *Process Biochem.* **1974**, *22*, 7–9.
4. Leathers, T. D. *Appl. Microbiol. Biotechnol.* **2003**, *62*, 468–473.
5. Isaka, M.; Rugseree, N.; Maithip, P.; Kongsaeree, P.; Prabpai, S.; Thebtaranonth, Y. *Tetrahedron* **2005**, *61*, 5577–5583.
6. Isaka, M.; Prathumpai, W.; Wongsa, P.; Tanticharoen, M. *Org. Lett.* **2006**, *8*, 2815–2817.
7. Seephonkai, P.; Kongsaeree, P.; Prabpai, S.; Isaka, M.; Thebtaranonth, Y. *Org. Lett.* **2006**, *8*, 3073–3075.
8. Boss, R.; Scheffold, R. *Angew. Chem., Int. Ed. Engl.* **1976**, *15*, 558–559.
9. Since the reaction conditions for cleavage of allyl ethers were employed, it is not certain whether palladium took part in this deprenylation reaction. It should also be noted that the yield of **5** dropped when the experiment was repeated in a similar scale.
10. Isaka, M.; Palasarn, S.; Sriklung, K.; Kocharin, K. *J. Nat. Prod.* **2005**, *68*, 1680–1682.
11. Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591–596.
12. Bhushan, R.; Brückner, H. *Amino Acids* **2004**, *27*, 231–247.
13. Desjardins, R. E.; Canfield, C. J.; Chulay, J. D. *Antimicrob. Agents Chemother.* **1979**, *16*, 710–718.
14. Collins, L.; Franzblau, S. G. *Antimicrob. Agents Chemother.* **1997**, *41*, 1004–1009.
15. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107–1112.