

## ORIGINAL ARTICLE

# *In vitro* antitrypanosomal activity of the cyclodepsipeptides, cardinalisamides A–C, from the insect pathogenic fungus *Cordyceps cardinalis* NBRC 103832

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During the search for new antitrypanosomal drug leads, three new antitrypanosomal compounds, cardinalisamides A–C (1–3), were isolated from cultures of the insect pathogenic fungus *Cordyceps cardinalis* NBRC 103832. Their structures were elucidated using MS analyses and extensive 2D-heteronuclear NMR. The absolute configurations of 1–3 were addressed by chemical degradation and Marfey's analysis. 1–3 showed *in vitro* antitrypanosomal activity against *Trypanosoma brucei brucei* with IC<sub>50</sub> values of 8.56, 8.65 and 8.63  $\mu\text{g ml}^{-1}$ , respectively.

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## INTRODUCTION

Human African trypanosomiasis, also known as sleeping sickness, is a potentially fatal parasitic disease transmitted by the bite of the tsetse fly that plagues many regions of Africa. Although the number of people being infected with the disease has declined due to better diagnosis and treatment, there were still more than 7000 new cases recorded in 2010.<sup>1</sup> Without treatment, sleeping sickness is fatal. Moreover current therapies often have unpleasant side effects.

During the course of our screening program for compounds that treat the disease, which is caused by the parasite *T. brucei*, we have reported on various natural products, such as microbial metabolites and plant products that exhibit potent antitrypanosomal properties.<sup>2–8</sup> This time we focused on metabolites from cultures of *Cordyceps* that showed antitrypanosomal activity. *Cordyceps* is a fungal genus which is endoparasitic with arthropods, principally insects. This genus is proving to be a rich source of novel secondary metabolites including important compounds for pharmaceutical discovery.<sup>9–14</sup>

In 2004, Professor Joseph W. Spatafora reported on *C. cardinalis*, a pathogen of lepidopteran larvae, a new species from the southern Appalachian Mountains of the eastern United States and from southeastern Japan.<sup>15</sup> *C. cardinalis* NBRC 103832 was isolated from single ascospores of a fruiting body occurring on a lepidopteran larva from Japan (Takatsuki, Osaka). The single stroma is ochraceous orange to red and less than 30 mm. The anamorphic stage on PDA is

Mariannaea-like, producing imbricate conidia chains. Sequence analysis of this strain based on rDNA ITS1–5.8S–ITS2 showed 99% similarity with *C. cardinalis* BCMU CC01 (AB237660).

Here, we report the isolation, structural determination and antitrypanosomal activity of three depsipeptides, cardinalisamides A–C (1–3).

## RESULTS

### Extraction and isolation

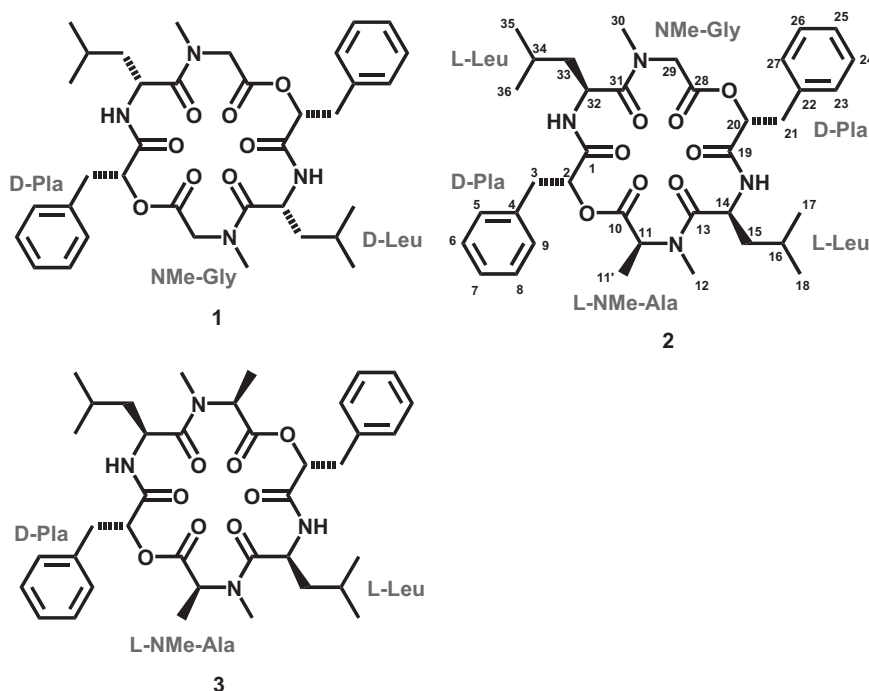
Laboratory cultures of *C. cardinalis* NBRC 103832 were grown as lawns on solid agar. The mycelia were harvested and extracted exhaustively with MeOH. The mycelial extract was evaporated to an aqueous concentrate and successively extracted with EtOAc. The extracted material (7.1 g) was chromatographed on silica gel by *n*-hexane and an increasing ratio of CHCl<sub>3</sub> and by preparative HPLC using 70–75% MeOH to afford three new compounds, cardinalisamides A–C (1–3) (Figure 1).

### Structure elucidation

Cardinalisamide A (1) was obtained as an oil, for which analysis based on HRFABMS data ( $m/z$  687.3373 for  $[\text{M} + \text{Na}]^+$ ) gave the molecular formula C<sub>36</sub>H<sub>48</sub>N<sub>4</sub>O<sub>8</sub>. The <sup>1</sup>H and COSY NMR spectra (Table 1) showed typical features for peptides, with an amide proton signal ( $\delta_{\text{H}}$  7.49), an *N*-methyl group ( $\delta_{\text{H}}$  3.27) and four  $\alpha$  protons

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**Figure 1** Structures of cardinalisamides A–C (1–3). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

( $\delta_{\text{H}}$  5.60, 5.23, 4.43, 3.18). The  $^{13}\text{C}$  NMR and HSQC spectra and a DEPT experiment indicated three carbonyl carbons, six olefinic carbons, three methines, three methylenes, one downfield methyl at 37.7 ppm and two upfield methyl signals. Based on the carbon numbers in  $^{13}\text{C}$  NMR and the molecular formula, **1** possesses  $\text{C}_2$  symmetry. Interpretation of COSY, HSQC and HMBC spectra allowed three distinct fragments to be constructed (Figure 2). These included two phenyllactic acid (Pla) moieties and amino acid moieties, such as two *N*-methylglycines (*N*-MeGly) and two leucines (Leu). The sequence of **1** was assigned by HMBC analysis using correlations from the  $\alpha$ -protons or the *N*-methyl protons to the carbonyl carbon resonances. The linkage of *N*-MeGly and Pla was established by HMBC correlations from the  $\alpha$ -proton of Pla ( $\delta_{\text{H}}$  5.60) to the *N*-MeGly carbonyl ( $\delta_{\text{C}}$  166.7). Connection of Leu and Pla was determined on the basis of the HMBC correlation from the  $\alpha$ -proton of Leu ( $\delta_{\text{H}}$  5.23) to the Pla carbonyl ( $\delta_{\text{C}}$  168.4). Further, an HMBC correlation from the *N*-methyl singlet proton (H-12,  $\delta_{\text{H}}$  5.23) to the Leu carbonyl ( $\delta_{\text{C}}$  173.9) revealed the linkage of *N*-MeGly to Leu. Intense NOESY correlations from the 14-NH to H-2' and from the *N*-methyl protons to H-14 strongly suggested an alternate linkage of two amide bonds. Finally, the closure of the ring allowed the planar structure of cardinalisamide A (**1**) to be assigned.

The absolute configuration of Pla in **1** was determined as D by chiral HPLC analyses using direct comparison with authentic specimens. The absolute configuration of the amino acid (Leu) in **1** was assigned by application of Marfey's method.<sup>16–18</sup> Acid hydrolysis of the hydrogenated product of **1** yielded the expected free amino acids, which were derivatized using FDAA (Marfey's reagent, 1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide) and analyzed by LC/MS by comparing the chromatograms with those derivatives of authentic specimens. Retention times (in min) of the FDAA derivatives of Leu were L-Leu (16.6) and D-Leu (17.8). The amino acids (Leu  $\times$  2) in **1**, which had a retention time of 17.4, thus have the D-configuration.

The same extract afforded cardinalisamide B (**2**) as an oil, which was assigned the molecular formula  $\text{C}_{37}\text{H}_{50}\text{N}_4\text{O}_8$  based on HRFABMS data

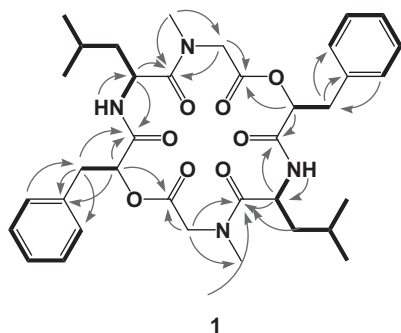
( $m/z$  701.3534 for  $[\text{M} + \text{Na}]^+$ ). This indicated that **2** differed from **1** by the presence of a  $\text{CH}_2$  unit. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **2** (Table 1) were similar to those of **1** except for  $\text{C}_2$  symmetry in **1**. COSY, HSQC and HMBC spectral data allowed the assignment of one *N*-MeGly, one *N*-methylalanine (*N*-MeAla), two Leu and two Pla. The sequence of **2** was also determined by HMBC spectra and NOE correlations between H-12 and H-14, 14-NH and H-20, H-30 and H-32 and 32NH and H-2. The absolute configuration of the amino acids and the Pla was deduced as follows. Acid hydrolysis and FDAA derivatization of Leu (16.7) revealed L-Leu by LC-MS analysis. The absolute configuration of *N*-MeAla was determined using the advanced Marfey method (using an FDLA derivative) because the retention times of the FDAA derivatives of D- and L-*N*-MeAla were very similar. FDLA derivatization of *N*-MeAla (9.2) revealed L-*N*-MeAla by LC-MS analysis. The configuration of the Pla was determined to be D, based on comparison of the retention time of the Pla from the hydrolysate with those of authentic specimens of D- and L-Pla on a chiral HPLC column.

Cardinalisamide C (**3**) was obtained as an oil that showed an  $[\text{M} + \text{Na}]^+$  peak at  $m/z$  715.3712, for a molecular formula of  $\text{C}_{38}\text{H}_{52}\text{N}_4\text{O}_8$  by HRFABMS, which differs from that of **2** by the presence of a  $\text{CH}_2$  unit. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **3** (Table 1) were similar to those of **2**. **3** possessed  $\text{C}_2$  symmetry based on its carbon numbers in the  $^{13}\text{C}$  NMR and the molecular formula. The difference in **3** was the replacement of an *N*-MeGly unit in **2** with an *N*-MeAla unit. Analysis of  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, HSQC, COSY and HMBC spectra revealed the presence of two *N*-MeAla, two Leu and two Pla moieties. Finally, the sequence of **3** was established by HMBC correlations and supported by observed NOE enhancements. The absolute configurations of the amino acid units of **3** were determined in an identical fashion to that used for **2**. Acid hydrolysis and L-FDAA derivatization of Leu (16.6) and L-FDLA derivatization of *N*-MeAla (9.2) revealed L-*N*-MeAla and L-Leu by LC-MS analysis, respectively. Finally, chiral-phase HPLC revealed L-Pla, completing the absolute configurational assignment of cardinalisamide C (**3**).

**Table 1** NMR data for cardinalisamides A (1)–C (3) in CDCl<sub>3</sub>

2			1		
Position	$\delta_C$ (m)	$\delta_H$ J (Hz)	Position	$\delta_C$ (m)	$\delta_H$ J (Hz)
1	168.8 (s)	5.40 1H, dd, 11.8, 3.0	1, 19	168.4 (s)	5.60 1H, dd, 11.4, 3.2
2	74.8 (d)		2	74.1 (d)	
3	38.7 (t)	2.75 2H, dd, 14.2, 11.3	3	38.6 (t)	3.63 1H, dd 14.1, 3.2
4	136.7 (s)		4	136.0 (s)	2.74 1H, dd 14.1, 11.4
5, 9	129.1 (d)	7.14 2H, brd, 8.0	5, 9	129.2 (d)	7.15 2H, brd, 8.0
6, 8	128.5 (d)	7.27 2H, m	6, 8	128.6 (d)	7.26 2H, m
7	127.1 (d)	7.21 1H, m	7	127.1 (d)	7.21 1H, m
10	169.3 (s)	3.15 1H, q, 6.9	10	166.7 (s)	4.43 1H, d, 17.2
11	59.7 (d)		11	51.7 (t)	
11'	13.5 (q)	1.41 3H, d, 6.9	12	37.7 (q)	3.18 1H, d, 17.2
12	38.0 (q)	3.37 3H, s			3.27 3H, s
13	172.3 (s)	5.15 1H, dt, 9.3, 7.6	13	173.9 (s)	5.23 1H, dt, 9.5, 7.6
14	46.2 (d)		14	46.1 (d)	
15	40.55 (t)	1.73 2H, m	15	40.9 (t)	1.77 2H, m
16	24.9 (d)	1.56 1H, m	16	24.7 (d)	1.54 1H, m
17	22.7 (q)	0.94–0.95 3H, m	17	22.6 (q)	0.94 3H, d, 6.6
18	22.8 (q)	0.94–0.95 3H, m	18	22.7 (q)	0.95 3H, d, 6.6
			14-NH		7.49 1H, d, 9.5
			3		
19	168.4 (s)	5.61 1H, dd, 11.3, 3.3	1	168.8 (s)	5.41 1H, dd, 11.7, 3.0
20	73.9 (d)		2	74.6 (d)	
21	38.7 (t)	3.62 2H, m	3	38.6 (t)	3.61 1H, dd, 14.1, 2.9
22	136.0 (s)		4	136.7 (s)	2.76 1H, dd, 14.1, 11.8
23, 27	129.1 (d)	7.14 2H, brd, 8.0	5, 9	129.1 (d)	7.14 2H, brd, 7.0
24, 26	128.5 (d)	7.27 2H, m	6, 8	128.5 (d)	7.26 2H, m
25	127.0 (d)	7.21 1H, m	7	126.9 (d)	7.20 1H, m
28	166.7 (s)		10	169.3 (s)	
29	51.7 (t)	4.42 1H, d, 17.2	11	59.7 (d)	3.13 1H, q, 6.9
30	37.7 (q)	3.16 1H, d, 17.2	11'	13.5 (q)	1.40 3H, d, 6.9
		3.26 3H, s	12	38.0 (q)	3.36 3H, s
31	174.0 (s)	5.22 1H, dt, 9.5, 7.8	13	172.4 (s)	5.15 1H, dt, 9.3, 7.6
32	46.1 (d)		14	46.2 (d)	
33	40.58 (t)	1.82 2H, m	15	40.4 (t)	1.78 2H, dd, 7.4, 7.4
34	24.5 (d)	1.56 1H, m	16	24.7 (d)	1.56 1H, m
35	22.56 (q)	0.94–0.95 3H, m	17	22.6 (q)	0.93 3H, d, 6.6
36	22.63 (q)	0.94–0.95 3H, m	18	22.7 (q)	0.94 3H, d, 6.5
13-NH		7.79 1H, d, 9.6	14-NH		7.78 1H, d, 9.5
14-NH		7.47 1H, d, 9.5			

1 and 3 possessed C<sub>2</sub> symmetry.



**Figure 2** COSY and selected HMBC correlations for **1**, indicating the sequence of six residues. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

### Biological activity

Cardinalisamides A–C (**1**–**3**) showed *in vitro* antitrypanosomal activity against *T. brucei brucei* with IC<sub>50</sub> values of 8.56, 8.65 and 8.63  $\mu\text{g ml}^{-1}$ , and cytotoxicity against normal human diploid fibroblasts (MRC-5 cells) with IC<sub>50</sub> values of 18.48, 14.00 and 23.84  $\mu\text{g ml}^{-1}$ , respectively.

### EXPERIMENTAL SECTION

#### General experimental procedures

Optical rotations were recorded on a JASCO P-1030 polarimeter. IR spectra were measured on a Shimadzu FTIR-8400S instrument. NMR spectra were obtained on a Varian UNITY 600 NMR spectrometer. The chemical shifts are given in  $\delta$  (ppm), and coupling constants are reported in Hz. HRMS spectra were obtained on a JEOL JMS-700 instrument. Kieselgel 60 (230–400 mesh, Merk) was used for column chromatography, and silica gel 60 F-254 (Merk)

for TLC. HPLC was performed on a JASCO-PU 1580 instrument with a COSMOSIL C18 P-Ms II (250 × 20 mm).

### Fungal material, extraction and isolation

The fungus *C. cardinalis* NBRC 103832 was identified by Dr Ban of the Nite Biological Resource Center (NBRC). A voucher specimen (*C. cardinalis* NBRC 103832) is deposited at the NBRC culture Collection. *C. cardinalis* NBRC 103832 was cultured for 2 weeks at 25°C in potato sucrose medium (10 l culture). Mycelia were separated from the broth by filtration and subsequently extracted twice with MeOH to yield 16.3 g of a crude extract in an ultrasonic bath for 60 min. The extract was concentrated and the resulting residue was extracted with EtOAc (500 ml × 3). The EtOAc-soluble portion (7.1 g) was repeatedly subjected to silica gel flash column chromatography (using increasing concentrations of CHCl<sub>3</sub> and MeOH in n-hexane as eluent). Further purification was carried out by repeated preparative HPLC on an ODS C-18 column, using MeOH and H<sub>2</sub>O as a solvent system to afford 1 (12.6 mg), 2 (53.6 mg) and 3 (34.9 mg).

Cardinalisamide A (1): pale yellow oil;  $[\alpha]_D^{23}$  –46.6° (c 0.33, MeOH); FT-IR (film) cm<sup>–1</sup>: 3299, 1751, 1672, 1636, 1626; HRFABMS *m/z*: [M + Na]<sup>+</sup> 687.3373, calcd for C<sub>36</sub>H<sub>48</sub>N<sub>4</sub>O<sub>8</sub>Na 687.3370; COSY and HMBC data see Figure 1.; Selected NOESY (H/H) 12/14, 14-NH/20.

Cardinalisamide B (2): pale yellow oil;  $[\alpha]_D^{23}$  –86.8° (c 0.59, MeOH); FT-IR (film) cm<sup>–1</sup>: 3279, 1753, 1672, 1636, 1628; HRFABMS *m/z*: [M + Na]<sup>+</sup> 701.3534, calcd for C<sub>37</sub>H<sub>50</sub>N<sub>4</sub>O<sub>8</sub>Na 701.3526; COSY (H/H) 2/3, 5/6, 6/7, 7/8, 8/9, 11/11', 14NH/14, 14/15, 15/16, 16/17, 16/18, 20/21, 23/24, 24/25, 25/26, 26/27, 32NH/32, 32/33, 33/34, 34/35, 34/36; HMBC (H/C) 2/1, 2/10, 3/5, 3/4, 3/9, 5/3, 9/3, 11/10, 11/12, 11/13, 12/11, 12/13, 13/12, 14/13, 14/19, 20/19, 20/28, 21/22, 21/23, 21/27, 23/21, 27/21, 29/28, 29/30, 29/31, 30/29, 30/31, 32/31, 32/1, 32NH/1; Selected NOESY (H/H) 12/14, 14-NH/20, 30/32, 32-NH/2.

Cardinalisamide C (3): pale yellow oil;  $[\alpha]_D^{23}$  –105.4° (c 0.84, MeOH); FT-IR (film) cm<sup>–1</sup>: 3275, 1751, 1672, 1659, 1629; HRFABMS *m/z*: [M + Na]<sup>+</sup> 715.3712, calcd for C<sub>38</sub>H<sub>52</sub>N<sub>4</sub>O<sub>8</sub>Na 715.3683; COSY (H/H) 2/3, 5/6, 6/7, 7/8, 8/9, 11/11', 14NH/14, 14/15, 15/16, 16/17, 16/18; HMBC (H/C) 2/1, 2/4, 3/1, 3/4, 3/5, 5/6, 5/7, 7/5, 11/10, 11/12, 11/13, 11'/10, 12/11, 12/13, 14/13, 14/1, 15/13; Selected NOESY (H/H) 5 or 9/11, 12/14, 14-NH/2'.

### Acid hydrolysis of 1–3

A tube containing 1–3 (1.2 mg) and 6 M HCl (1 ml) was kept at 110°C for 21 h. This solution was evaporated to dryness under vacuum to give the hydrolysate of 1–3, respectively.

### Absolute configuration of Pla in 1–3

The hydrolysate was dissolved in 1 ml of water, with 5 µl of this solution used for one analysis. HPLC analysis was performed on DAICEL OD-RH chiral column (250 × 4.6, 5 µm; solvents: (A) pH 2 formic acid + water, (B) 20% MeCN; 0.5 ml per min; 25°C). The configuration was determined by co-injection with L-Pla (tR 8.8) and D-Pla (tR 8.5).

### Preparation and analysis of Marfey derivatives

1–3 (0.2 mg) was hydrolyzed by heating in HCl (6 M; 1 ml) at 110°C for 14 h. After cooling, the solution was lyophilized and redissolved in 0.1 N HCl (100 µl). To the acid hydrolysate solution (or to 50 µl of a 50 mM solution of the respective amino acid) was added a 1% (w/v) solution (100 µl) of FDAA<sup>9</sup> or FDLA<sup>10</sup> in acetone. After addition of NaHCO<sub>3</sub> solution (1 M; 20 µl) the mixture was incubated for 1 h at 40°C. The reaction was stopped by addition of HCl (1 M; 20 µl), the solvents were evaporated to dryness, and the residue was redissolved in acetonitrile (100 µl). An aliquot of this solution (20 µl) was analyzed by HPLC (COSMOSIL 5C18 MS-II, 150 × 4.6, 5 µm; solvents: (A) 0.05% TFA water, (B) 0.05% TFA MeCN; linear gradient of from 10% MeCN to 70% MeCN in 15 min and isocratic at 70% MeCN for the next 10 min.; 0.5 ml min<sup>–1</sup>; 25°C). Retention times (min) of the FDAA derivatives of Leu were as follows: L-Leu (16.6) and D-Leu (17.8).

(COSMOSIL 5C18 MS-II, 150 × 4.6, 5 µm; solvents: (A) water + pH 2 formic acid, (B) MeCN; 40% B; 0.5 ml min<sup>–1</sup>; 25°C). Retention times (min)

of the FDLA derivatives of N-MeAla were as follows: L-N-MeAla (9.2) and D-N-MeAla (11.7).

### Trypanosome

The bloodstream forms of *T. b. brucei* strain GUTat 3.1 parasites were used for experimentation as described previously.<sup>19</sup>

### In vitro assay

The *in vitro* antitrypanosomal assays using *T. b. brucei* strain GUTat 3.1 have been described previously.<sup>19</sup> In brief, 95 µl of parasite suspension was incubated with 5 µl of drug solution for 72 h and Alamar Blue was used for parasite survival determination to calculate IC<sub>50</sub> values.

Cytotoxicity assay against human diploid embryonic cell line MRC-5 was carried out as previously described.<sup>20</sup>

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