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Circinamide, a Novel Papain Inhibitor from the Cyanobacterium *Anabaena circinalis* (NIES-41)

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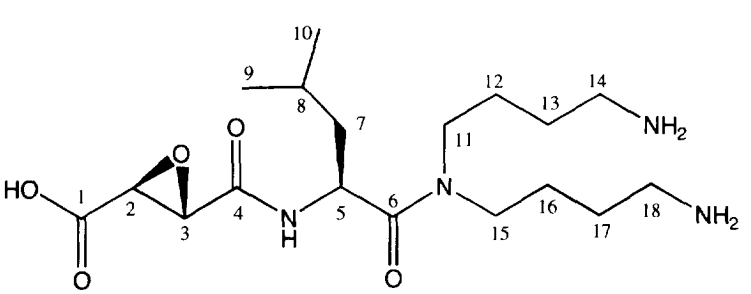
Abstract: Circinamide (**1**), a novel papain inhibitor, was isolated from the cyanobacterium *Anabaena circinalis* Rabenhorst (NIES-41). Its structure has been determined by a combination of 2D NMR data and chemical degradation and confirmed by mass spectral analysis. The absolute stereochemistry of **1** was determined by GC and HPLC analyses of degradation products of **1**. Circinamide (**1**) exhibited potent papain inhibitory activity with an IC_{50} of 0.4 μg/mL.

In screening extracts of laboratory-cultured cyanobacteria for enzyme inhibitors, the hydrophilic extract of *Anabaena circinalis* Rabenhorst (NIES-41) was found to exhibit selective inhibitory activity against papain. Assay-guided fractionation of the extract by reversed-phase flash column chromatography led to two fractions which were responsible for the papain inhibitory activity. The fractions were combined and purified by reversed-phase HPLC to give a novel papain inhibitor, named circinamide (**1**).

A number of serine and cysteine protease inhibitors have been isolated until now,[1] but only a few papain inhibitors are known to date. Actually, circinamide is the first papain inhibitor isolated from cyanobacteria. We report here the isolation and gross structure determination of circinamide which inhibits papain potently.



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Results and Discussion

Isolation. *A. circinalis* strain NIES-41 was isolated from Lake Kasumigaura, Japan,[2] and grown in mass culture in the laboratory by using the conditions described for *Oscillatoria agardhii*.[3] The 80% methanol extract of the freeze-dried cells (140 g) was fractionated by ODS flash column chromatography. The active fractions eluted with 40 and 50% MeOH were purified by reversed-phase HPLC on an ODS column to yield circinamide (**1**) as colorless microcrystals (13.5 mg, 0.01% yield).

Gross Structure Determination. Circinamide (**1**) had the molecular formula $C_{18}H_{34}O_5N_4$, as determined by HRFAB-MS [*m/z* 387.2612 $(M + H)^+$ Δ +0.4 mmu] and NMR data. The UV spectrum showed the maximum absorption at 250 nm with a relatively small molar absorptivity (ε 530). Interpretation of the ^1H and ^{13}C NMR spectral data (Table 1) of **1** indicated the presence of three carbonyls, one epoxide, two methines, nine methylenes, and two methyls. A detailed analysis of the 2D NMR data, including COSY, HMQC,[4] HMBC,[5] ROESY,[6] and HOHAHA[7] spectra gave partial structures **A-C**, viz., 2, 3-epoxy succinic acid, leucine, and *N*-(4-aminobutyl)-1, 4-butanediamine (homospermidine) (Fig. 1).

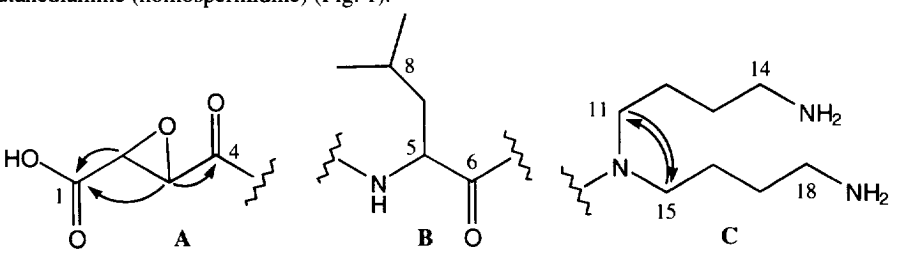
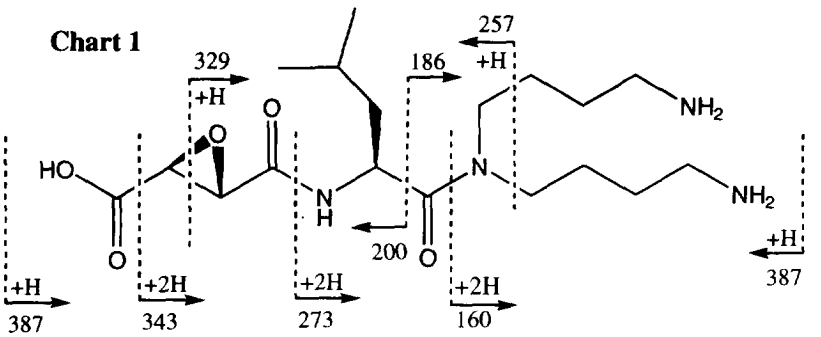


Fig. 1. Partial structures **A-C** of circinamide (**1**) with important HMBC correlations.

In the COSY spectrum, the C-2 oxygenated methine proton (δ 3.43) was correlated to the C-3 oxygenated methine proton (δ 3.63). Connectivity of C-1 to C-2 was inferred from the HMBC experiment, in which cross peaks were observed between H-2 and H-3 methine protons and C-1 carbonyl carbon. The HMBC spectrum also showed the correlation between H-3 and C-4 quaternary carbon at 165.1 ppm. The presence of epoxy group at the 2, 3-position was determined from the chemical shifts of proton (δ 3.43, 3.63) and carbon (δ 51.3, 52.4) signals of this position and the relatively large $^1J_{CH}$ values for C-2 ($^1J_{CH}$=188 Hz) and C-3 ($^1J_{CH}$=184 Hz).[8] All of these data confirmed the presence of 2, 3-epoxy succinic acid (Esa). In the positive FAB-MS, the fragment ion peak at *m/z* 273 $(M - Esa + 2H)^+$ also supported the presence of Esa (Chart 1).

Chart 1



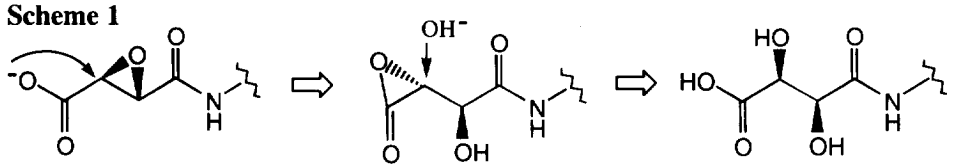
The peptidic nature of **1** was suggested by the ^{1}H and ^{13}C NMR spectra, and standard amino acid analysis revealed that Leu was the only amino acid in **1**. The homospermidine unit was assigned from the COSY, HOHAHA, and HMBC data. The COSY and HOHAHA spectra revealed two spin systems: one was from C-11 to C-14, and the other was from C-15 to C-18. The chemical shifts of four terminal methylenes (C-11, 14, 15, 18) indicated that the carbons were adjacent to the nitrogen atom. Acetylation of **1** with acetic anhydride and pyridine gave a diacetyl derivative, which was confirmed by the quasi-molecular ion peak at *m/z* 471 $(M + H)^+$ in the positive FAB-MS, indicating the presence of two amines. In the COSY spectrum, C-14 and C-18 methylenes were coupled with the amines located at δ 7.78 (4H). In the HMBC spectrum, correlations were observed between H-11 and C-15 and between H-15 and C-11, indicating that the C-11 and C-15 methylenes must be attached at tertiary amide. These data fully supported the presence of homospermidine unit. The presence of homospermidine unit in **1** was also supported by the fragment ion peak at *m/z* 160 in the positive FAB-MS.

The HMBC and ROESY data summarized in Table 1 allowed the assembly of partial structures A-C into gross structure **1**. The respective two-bond HMBC correlation between the amide proton of Leu and carbonyl carbon (C-4, δ 165.1) and a three-bond correlation from α-proton of Leu to C-4 as well as ROESY correlation between H-3 and amide proton of Leu confirmed the connectivity between **A** and **B**. There were three-bond HMBC correlations between the carbonyl carbon (C-6, δ 171.0) and the methylene protons of H-11a,b and H-15a,b, which indicated the connectivity of **B** to **C** and completed the gross structure of **1**. The ROESY correlation between H-5 and H-11a,b supported the connectivity of **B** to **C** and confirmed the *cis* relationship between C-6 amide carbonyl and C-15 to C-18 unit. The gross structure deduced from NMR data was well supported by the fragment peaks in the positive ion FAB-MS (Chart 1).

The absolute configurations of the epoxide and Leu were determined by GC and HPLC analyses, respectively. The epoxide was shown to be *trans* by the coupling constant ($J_{2,3}$ = 2.1 Hz).[9] Cleavage of the epoxide in **1** by saponification with 2 N NaOH to 2, 3-diol, followed by hydrolysis with 6N hydrochloric acid gave D-tartaric acid in the GC analysis, which confirmed the

absolute stereochemistry of the epoxide as 2S, 3S. In this condition, *meso*-tartaric acid was not detected. We anticipated that, with the help of neighboring group participation, the stereoselectivity of the nucleophilic attack could be altered so that the nucleophile could approach from the same face as the leaving group, resulting in net retention of configuration (Scheme 1).

Scheme 1



The absolute stereochemistry of Leu was determined to be L-form by HPLC analysis of the acid hydrolysate derivatized with Marfey's reagent.[10]

Table 1. ^{1}H and ^{13}C NMR data of circinamide (**1**) in DMSO-d_6

No.	^{1}H (mult, J Hz)	^{13}C (mult)	HMBC[a] correlations	ROESY[b] correlations
1		168.7 (s)	H-2, H-3	
2	3.43 (d, 2.1)	51.3 (d)	H-3	
3	3.63 (d, 2.1)	52.4 (d)	H-2	5-NH
4		165.1 (s)	H-3, H-5, 5-NH	
5	4.70 (ddd, 11.5, 8.5, 3.5)	47.1 (d)	H-7a,b	5-NH, H-7a,b, H-9, H-11a,b
5-NH	8.72 (d, 8.5)			H-5, H-7a,b
6		171.0 (s)	H-5, H-11a,b, H-15a,b	
7a	1.30 (ddd, 11.5, 10.4, 4.2)	40.8 (t)	H-5, H-9, H-10	H-5, H-9, H-10
7b	1.58 (m)			H-5, H-9, H-10
8	1.58 (m)	24.3 (d)	H-5, H-7a,b, H-9, H-10	H-5, H-9, H-10
9	0.88 (d, 6.5)	21.2 (q)	H-7a,b, H-8, H-10	H-7a,b, H-8
10	0.89 (d, 6.5)	23.1 (q)	H-7a,b, H-8,H- 9	H-7a,b, H-8
11a	3.22 (m)	46.3 (t)	H-12a,b, H-15	H-5, H-12a,b
11b	3.30 (m)			H-5, H-12a,b
12a	1.52 (m)	25.7 (t)	H-11a,b, H-13, H-14	H-11a,b, H-14
12b	1.62 (m)			H-11a,b, H-14
13a,b	1.52 (m)	24.3 (t)	H-14	H-14
14a,b	2.80 (m)	38.6 (t)	H-12a,b, H-13	H-12a,b, 14-NH_2
14-NH_2	7.78 (br s)			H-14
15a	3.06 (dd, 13.5, 6.2)	44.5 (t)	H-11	H-16, H-17
15b	3.41 (m)			H-16, H-17
16a,b	1.46 (m)	24.1 (t)	H-15a,b, H-18	H-15a,b
17a,b	1.46 (m)	24.1 (t)	H-15a,b, H-18	H-15a,b, H-18
18a,b	2.78 (m)	38.6 (t)	H-16, H-17	H-16, H-17, 18-NH_2
18-NH_2	7.78 (br s)			H-18

[a]Optimized for $^{2,3}J_{CH}$ = 8.3 Hz. [b]Mixing time = 200 ms.

Circinamide (**1**) inhibited papain selectively with an IC_{50} of 0.4 μg/mL but had no inhibitory activity against thrombin, trypsin, chymotrypsin, plasmin, and elastase at 100 μg/mL. Circinamide

was found to have stronger inhibitory activity than the well-known papain inhibitors such as leupeptin (IC_{50} = 0.5 μg/mL)[11] and chymostatin (IC_{50} = 7.5 μg/mL),[12] but it showed weaker activity than antipain (IC_{50} = 0.2 μg/mL).[13]

Circinamide (**1**) is a novel papain inhibitor containing unusual acid and polyamine, named 2, 3-epoxy succinic acid and homospermidine, respectively. Spermidine is a naturally occurring polyamine and is present in ptilomycalin A[14] and crambescidins[15] as a structural unit of the major metabolites of sponge, while homospermidine is a relatively rare triamine.[16] Circinamide (**1**) is related to E-64, a potent cysteine protease inhibitor isolated from cultures of *Aspergillus japonicus*,[17] and E-64-c.[18] Based on the prototype E-64, loxistatin[19] was designed as a clinically usable drug for the treatment of muscular dystrophy[20] by exhaustive studies on the structure-activity relationship. To facilitate the design of more potent and useful protease inhibitors, it is desirable to establish the mode of binding of circinamide to papain, which will be the theme of our next study.

Experimental section

Instrumentation

NMR spectra were recorded on either a JEOL JNM-A600 or JEOL JNM-A500 NMR spectrometer, operating at 600 or 500 MHz for ^1H, respectively, using DMSO as solvent at 27 °C. ^1H and ^{13}C NMR chemical shifts were referenced to solvent peaks: δ_H 2.49 and δ_C 39.5 for DMSO-d_6. FAB mass spectra were measured by using glycerol or polyethyleneglycol as matrix on a JEOL JMS SX-102 mass spectrometer. Amino acid analysis was carried out with a Hitachi L-8500A amino acid analyzer. HPLC was performed on a Shimadzu LC-6A liquid chromatograph with an ODS L-column (10 × 250 mm, Chemicals Inspection and Testing Institute, Japan). Chiral GC experiments were performed on a Shimadzu GC-9A gas chromatograph fitted with a Chirasil-Val capillary column (25 m × 0.25 mm) with a FID. Ultraviolet spectrum was measured on a Hitachi 330 spectrometer. Optical rotations were determined with a JASCO DIP-1000 digital polarimeter.

Culture conditions

Anabaena circinalis Rabenhorst (NIES-41) was obtained from the NIES-collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan). A 1-L culture of the cyanobacterium was used to inoculate an autoclaved 10 L glass bottles containing CB medium.[21] Cultures were illuminated on a 12L:12D cycle at an intensity of 250 μE/m^2·s from fluorescent tubes and aerated with filtered air (0.3 L/min, without added CO_2) at 25 °C. Cells were harvested by continuous flow centrifugation at 10,000 rpm after 14-18 days. Harvested cells were lyophilized and kept in a freezer at −20 °C until extraction.

Extraction and isolation

Freeze-dried cells (140 g from 420 L of culture) were extracted three times with 80% MeOH and once with MeOH. The extracts were combined and concentrated to give a crude extract (32.3 g). This extract was partitioned between ether and water. The water soluble fraction was further partitioned between *n*-BuOH and water. The hydrophilic portion (20.5 g), which showed papain inhibitory activity at 10 μg/mL, was subjected to ODS flash chromatography (AM 120-230/70, YMC CO., LTD, 12 × 10.5 cm) and eluted with H_2O, 10-50% MeOH, MeOH, and CH_2Cl_2. The active 40 and 50% MeOH fractions, which accounted for essentially the papain inhibitory activity, were combined and evaporated to give bright yellow solid (346 mg). Final purification was achieved by reversed-phase HPLC on an ODS L-column (linear gradient of CH_3CN in H_2O containing 0.05% TFA, 5% to 50% in 50 min; flow rate 2.5 mL/min; UV detection at 210 nm) to yield circinamide (**1**, 13.5 mg, t_R: 22.4 min).

Circinamide (**1**): $[\alpha]_D^{20}$ +15.5° (*c* 0.1, MeOH); UV (MeOH) λmax 250 nm (ε 530); FAB-MS *m/z* 387 $(M + H)^+$, HRFAB-MS *m/z* 387.2612 $(M + H)^+$ calcd. for $C_{18}H_{35}O_5N_4$, Δ +0.4 mmu. ^1H and ^{13}C NMR, see Table 1.

Amino acid analysis

Compound **1** (100 μg) was dissolved in 6 N HCl (500 μL) and sealed in a reaction vial. The vial was heated at 100 °C for 16 h. The solution was evaporated in a stream of dry nitrogen with heating and redissolved in 0.1 N HCl to subject to amino acid analysis.

Derivatization of amino acid and HPLC analysis

A 100 μg portion of **1** was dissolved in 500 μL of 6 N HCl and heated at 110 °C for 16 h. After removal of HCl in a stream of dry nitrogen, the residue was treated with 10% acetone solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA, Marfey's reagent) in 1 M $NaHCO_3$ at 80-90 °C for 3 min followed by neutralization with 50 μL of 2 N HCl. The reaction mixture was dissolved in 50 % MeCN and subjected to reversed-phase HPLC: column; Cosmosil MS (Nacalai Tesque Co., 4.6 × 250 mm), gradient elution from H_2O/TFA (100:0.1) to MeCN/H_2O/TFA (50:50:0.1) in 50 min, flow rate 1 mL/min, UV (340 nm). Retention times (min) of standard amino acids were as follows: L-Leu (56.4), D-Leu (60.8). The retention time for the derivatized **1** was 56.4 min.

Acetylation

A mixture of 500 μg of **1** in each 200 μL of acetic anhydride and pyridine was allowed to stir overnight. The excess reagents were removed under reduced pressure and the residue was dissolved in 200 μL of aqueous CH_3CN, filtered, and purified by reversed-phase HPLC eluting with a linear gradient from 10 to 70% CH_3CN in 0.05% aqueous TFA at a flow rate of 0.6 mL/min.

The major product showed an ion peak in the positive FAB-MS at *m/z* 471, indicating the formation of a diacetate of **1**, which was not characterized further.

Conversion of epoxy to diol and GC analysis

To a solution of 200 μg of **1** in 500 μL of 4:1 acetone/water was added 10 μL of 2 N NaOH. After being stirred at room temperature for 5 h, the reaction mixture was neutralized with 1 N HCl and evaporated. The residue was dissolved in 500 μL of 6 N HCl and heated at 110 °C for 12 h. The solvent was removed under vacuum, and the hydrolysate was treated with a solution of 10% HCl in isopropyl alcohol at 100 °C for 30 min. The excess reagent was then evaporated at 80 °C under a stream of dry nitrogen, and the residue was treated with 600 μL of 1:1 $(CF_3CO)_2O/CH_2Cl_2$ at 100 °C for 5 min. The excess reagent was then evaporated in a stream of dry nitrogen, and the resulting residue was dissolved in 500 μL of CH_2Cl_2 for chiral GC analysis on a Chirasil-Val column (Alltech). Column temperature was held at 80 °C for 3 min after injection of sample and then increased from 80 °C to 200 °C at 4 deg/min. The same procedure was repeated for standard samples of tartaric acid. The retention times for authentic D-, L-, and meso-tartaric acid isopropyl esters were found to be 6.12, 6.28, and 7.23 min, respectively. The retention time for the derivatized **1** was 6.10 min.

Protease inhibitory activity assay

Serine and cysteine protease inhibitory activities were determined by the method previously described.[3b]

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