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Urukthapelstatin A, a Novel Cytotoxic Substance from Marine-derived *Mechercharimyces asporophorigenens* YM11-542

II. Physico-chemical Properties and Structural Elucidation

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Abstract The new cyclic peptide antibiotic, urukthapelstatin A, has been isolated from a culture of *Thermoactinomycetaceae* bacterium *Mechercharimyces asporophorigenens* YM11-542. The structure of urukthapelstatin A was elucidated by NMR, MS, Marfey analysis, chiral HPLC and X-ray crystal analyses.

Keywords urukthapelstatin A, cyclic peptide, cytotoxic, *Thermoactinomyces*

Introduction

Urukthapelstatin A (**1**) is a thiopeptide antibiotic which contains three oxazoles and two thiazoles in its cyclic structure and is structurally related to mechercharstatin (former name, mechercharmycin) [1, 2] and YM-216391 [3, 4]. The fermentation, isolation and biological properties of **1** (Fig. 1) have been reported in the preceding paper [5]. This report describes the physico-chemical properties and structural determination of **1**, which was produced by *Mechercharimyces asporophorigenens* YM11-542.

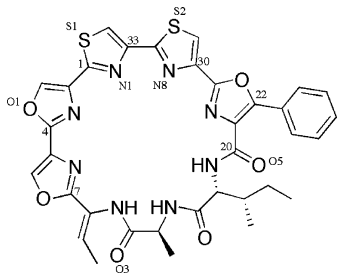

Fig. 1 Structure of urukthapelstatin A (**1**).

Results

Physico-chemical Properties

The physico-chemical properties of **1** are summarized in Table 1. **1** was isolated as a white solid. It was soluble in DMSO, DMF, $CHCl_3$ and CH_2Cl_2, poorly soluble in MeOH and MeCN, and insoluble in water. An analysis of low-resolution FAB-MS data in the positive and negative modes revealed its molecular mass to be 710. The molecular formula of **1** was established as $C_{34}H_{30}N_8O_6S_2$ by high-resolution FAB-MS data ($[M+Na]^+$: found, *m/z* 733.1616; calcd. for $C_{34}H_{30}N_8O_6S_2Na$, *m/z* 733.1627).

Structural Elucidation

In the ^{1}H-NMR spectrum, three methyl signals (δ_H 0.85, 0.86 and 1.50) and one vinyl methyl (δ_H 1.82) were observed. Five aromatic protons (δ_H 7.51, 2×7.55 and 2×8.34), four downfield singlets (δ_H 8.59, 8.69, 8.88 and 9.07), one olefinic proton (δ_H 6.62), and three exchangeable downfield proton signals (δ_H 8.75, 9.00 and 9.49) were also observed. The ^{13}C-NMR spectrum revealed 32 distinguishable carbon signals, of which there were 16 quaternary, 13 methine, 1 methylene and 4 methyl resonances. All of these 16 quaternary resonances were in

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Table 1 Physico-chemical properties of **1**

Appearance	White powder
MP (from CH_2Cl_2/MeOH)	311°C (decomp.)
Molecular formula	$C_{34}H_{30}N_8O_6S_2$
FAB-MS (*m/z*)	
Positive	711 $[M+H]^+$, 733 $[M+Na]^+$
Negative	709 $[M-H]^-$
HR-FAB-MS (*m/z*)	
Found	733.1616 $[M+Na]^+$
Calcd	733.1627
UV λ_{max}^{MeCN} nm (log ε)	267 (4.35), 291 (4.52)
IR (KBr) cm^{-1}	3393, 2921, 2851, 1654, 1507, 1459
$[\alpha]_D^{22}$	+38° (*c* 0.5, $CHCl_3$)

the δ_C 123~170 range, again suggesting a peptide with a highly aromatic character. Collectively, the molecular formula, and the UV, IR and NMR signals revealed the compound to be similar to mechercharstatin [1, 2] and YM-216391 [4] which contain oxazoles and a thiazole in their structures. In particular, there was significant overlap between the NMR data for the compound and the published data for mechercharstatin and YM-216391 [1, 2, 4].

The ^{1}H- and ^{13}C-NMR chemical shift assignments for **1** were based on 2D NMR analyses (COSY, TOCSY, HSQC, HMBC and ROESY) shown in Table 2. A detailed analysis of the ^{1}H-^{1}H COSY and TOCSY experiments revealed the partial structures of CH_3–CH=, CH_3–CHX–NH– and CH_3–CH_2–CH(–CH_3)–CHX–NH–, and a mono-substituted benzene moiety. The presence of oxazole and thiazole units was deduced by comparing the corresponding ^{1}H and ^{13}C chemical shifts with those of mechercharstatin and YM-216391. ^{1}H and ^{13}C chemical shifts and HMBC signals of **1** from aromatic protons to carbons; δ_H 9.07 to δ_C 135.50 and 155.11, δ_H 8.88 to δ_C 129.56 and 159.65, δ_H 8.69 to δ_C 141.93 and 161.08, and δ_H 8.59 to δ_C 147.79 and 157.26, suggest the existence of two oxazols and two thiazols in the structure.

The relationship of the spin networks was established from ^{1}H-^{13}C long-range correlations in the HMBC spectrum and yielded three partial structures, which were assigned to a bithiazole-benzyloxazole moiety ($C_{15}H_7N_3OS_2$, partial structure **A**), one oxazole (C_3HNO, partial structure **B**), and an alanine and isoleucine-containing part ($C_{16}H_{22}N_4O_4$, partial structure **C**, Fig. 2). The HMBC correlations when $^nJ_{CH}$ was set to 2 Hz, were observed from H-34 (δ_H 8.59) to C-2 (δ_C 135.50), and from H-6 (δ_H 8.88) to C-4 (δ_C 155.11). Consequently, C-1 of partial structure **A** was connected to C-2 of partial structure **B**, and C-4 of partial structure **B** was connected to C-6 of partial structure **C**. In addition, the ROESY correlation of H-10 (δ_H 1.82) and N4-H (δ_H 9.49) revealed that a vinylmethyl part in partial structure **C** had a *Z* configuration (Fig. 2).

Although there is no signal between partial structure **A** and **C** in HMBC spectrum, the C-20/C-21 connection can be brought about by the comparison of the molecular formula $C_{34}H_{30}N_8O_6S_2$ with a tolal of each partial structures (**A**; $C_{15}H_7N_3OS_2$, **B**; C_3HNO, **C**; $C_{16}H_{22}N_4O_4$), enabling the planer structure of **1** to be obtained.

The absolute configuration of the alanine and isoleucine residues was determined by a Marfey analysis [6] and chiral HPLC analysis of the acid hydrolysate of **1**. The Marfey analysis clarified the existence of L-Ala in the acid hydrolysate of **1**, but D-Ile and D-*allo*-Ile were indistinguishable from the reversed-phase HPLC analysis of their FDAA derivatives. The chiral HPLC analysis successfully defined the absolute configuration of isoleucine as D-*allo*-Ile. Finally, the absolute stereochemistry of **1** was determined as shown in Fig. 1.

An X-ray crystallographic analysis was also performed to confirm the full structure of **1**. An adequate crystal was obtained by crystallization from CH_2Cl_2/MeOH (1 : 1). The crystal data and measurement conditions are summarized in the experimental section, and the ORTEP drawing shown in Fig. 3. The absolute configuration of **1** was ascertained from an X-ray anomalous dispersion of the S atom as shown in Fig. 1.

Discussion

The structure of **1** was determined by spectroscopic methods, chemical degradation, and by an X-ray crystallographic analysis. The structure of **1** was

Table 2 ^{1}H- and ^{13}C-NMR data for **1**

Position	^{13}C δ, mult.	^{1}H δ, mult., J (Hz)	HMBC
1	157.26, s		
2	135.50, s		
3	139.43, d	9.07, s	C-2, C-4
4	155.11, s		
5	129.56, s		
6	140.12, d	8.88, s	C-4*, C-5, C-7
7	159.65, s		
8	123.70, s		
9	128.78, d	6.62, q, 7.5	C-7, C-8, C-10
10	13.38, q	1.82, d, 7.5	C-7, C-8, C-9
11	170.30, s		
12	48.13, d	4.75, dq, 6.8, 7.5	C-11, C-13
13	19.80, q	1.50, d, 6.8	C-11, C-12
14	169.45, s		
15	58.45, d	4.52, dd, 8.3, 9.0	C-16, C-17, C-18, C-20
16	36.92, d	2.06, m	C-14, C-15, C-17, C-18, C-19
17	15.09, q	0.85, d, 6.8	C-15, C-16, C-18
18	25.24, t	1.09, m and 1.46, m	C-15, C-17, C-19
19	11.09, q	0.86, t, 7.5	C-16, C-18
20	159.95, s		
21	130.52, s		
22	151.00, s		
23	126.64, s		
24	127.78, d	8.34, d, 7.5	C-21*, C-22, C-26, C-28
25	128.30, d	7.55, dd, 6.8, 7.5	C-23, C-27
26	129.84, d	7.51, dd, 6.8, 6.8	C-24, C-28
27	128.30, d	7.55, dd, 6.8, 7.5	C-23, C-25
28	127.78, d	8.34, d, 7.5	C-21*, C-22, C-24, C-26
29	154.02, s		
30	141.93, s		
31	123.05, d	8.69, s	C-29, C-30, C-32
32	161.08, s		
33	147.79, s		
34	121.06, d	8.59, s	C-1, C-2*, C-32, C-33
N4-H	—	9.49, br s	C-7, C-8, C-9, C-11
N5-H	—	8.75, br d, 7.5	C-12, C-13, C-14
N6-H	—	9.00, br	

* HMBC correlations observed only when $^{n}J_{CH}$ was set to 2 Hz.

characterized as a cyclic peptide containing L-Ala, D-*allo*-Ile and a unique bioxazole-bithiazole-phenyloxazole moiety. Compound **1** is structurally related to the 24-membered cyclic compound, including mechercharstatin isolated from another *Thermoactinomycetaceae* bacterium [1, 2], YM-216391 from *Streptomyces nobilis* [3, 4] and telomestatin that has been isolated from *Streptomyces anulatus* [7]. This class of cyclic peptides containing a sequential oxazole-thiazole moiety represents a new group of antibiotics with highly potent activities. Further studies describing methods to improve the pharmaceutical properties of **1** will be reported elsewhere.

Experimental

General Procedures

Melting point (mp) values were determined with micro-

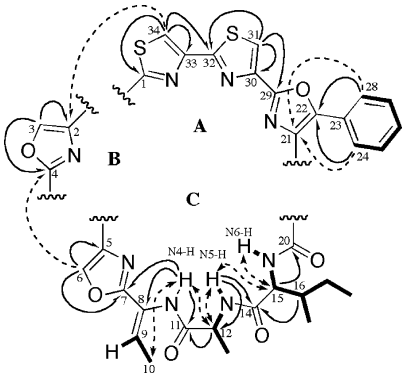

Fig. 2 2D NMR spectral data and partial structures of **1**.

—, COSY and TOCSY; ⌒, HMBC; ⌒, HMBC ($^nJ_{CH}$=2 Hz); ⌒, ROESY.

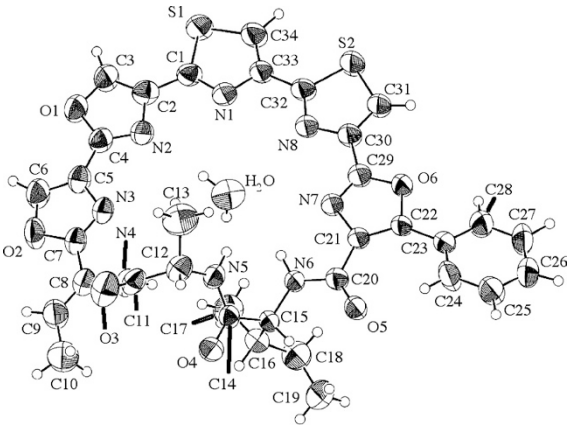

Fig. 3 ORTEP drawing for **1**.

One of two molecules of urukthapelstatin A in a unit cell and co-crystallized solvent molecules are omitted for clarity.

melting point apparatus (Yanaco). Optical rotation values were measured with a SEPA-300 digital polarimeter (Horiba), and IR spectra were obtained with an FT/IR-430 infrared spectrometer (Jasco). NMR (500 and 750 MHz for ^{1}H) spectra were obtained with a Unity INOVA NMR spectrometer (Varian), and low- and high-resolution mass spectra were measured with a JMS-SX102A instrument (Jeol). The X-ray diffraction analysis was carried out on a Mac Science (Bruker Nonius) dip image plate diffractometer, using graphite-monochromated Mo K_α radiation (λ=0.71073 Å).

Marfey Analysis

Approximately 200 μg of **1** was hydrolyzed with 500 μl of 6 N HCl at 120°C for 16 hours. The solution was freeze-dried and partitioned between $CHCl_3$ and water. The aqueous layer was then freeze-dried and used for the Marfey analysis [6]. A sample and standard amino acids (L-Ala, DL-Ala, L-Ile and DL-Ile, 2 nmol each) were dissolved in 100 μl of 0.1 N $NaHCO_3$, and 50 μl of a 0.5% FDAA-acetone solution (1-fluoro-2,4-dinitrophenyl-5-L-alanineamide, Marfey's reagent, Pierce) was added. The mixture was heated at 65°C for 10 minutes, cooled to room temperature, and 50 μl of 0.2 N HCl was added. Each 100 μl of the FDAA-derived mixture was directly analyzed by HPLC. The analysis of the FDAA derivatives was performed in a TSKgel ODS-80Ts column (150×4.6 mm, Tosoh Co.) maintained at 30°C with UV detection at 340 nm. MeOH, 0.1% TFA containing MeCN and 0.1% TFA containing water were used as the mobile phase in the linear-gradient elution mode (constant 25% MeOH, 0.1% TFA containing 10~50% MeCN for 22 minutes) at a flow rate of 1.0 ml/minute. The retention times of the FDAA derivatives of the amino acid hydrolysate were 11.55 minutes (L-Ala; D-Ala=14.61 minutes) and 20.91 minutes (D-Ile and D-*allo*-Ile; L-Ile and L-*allo*-Ile=17.79 minutes).

Chiral HPLC Analysis

To determine the absolute configuration of Ile, another hydrolysate of **1** was prepared. Approximately 500 μg of **1** was hydrolyzed with 1.0 ml of 6 N HCl at 117°C for 15 hours. The solution was partitioned three times between ethyl acetate and water. The aqueous layer was freeze-dried and dissolved in 200 μl of water, and 50 μl of the hydrolysate was analyzed by chiral HPLC in a Sumichiral OA-5000 column (150×4.6 mm, Sumika Chemical Analysis Service) maintained at 30°C with UV monitoring at 254 nm, using 5% i-propanol containing 2 mM $CuSO_4$ as the mobile phase at a flow rate of 1.0 ml/minute. The retention time of the acid hydrolysate of **1** was 12.32 minutes (D-*allo*-Ile; D-Ile=15.83 minutes).

X-Ray Crystal Analysis

A suitable crystal was obtained by crystallization from CH_2Cl_2/MeOH (1 : 1). The ORTEP drawing is shown in Fig. 3. The absolute configuration of **1** was determined from an X-ray anomalous dispersion of the S atom as shown in Fig. 3.

The crystal used for the X-ray crystallographic analysis contained 3 molecules of CH_2Cl_2, 2 molecules of H_2O and 2 molecules of **1** in a unit cell. Crystal data for **1**: $2(C_{34}H_{30}N_8O_6S_2)\cdot 2(H_2O)\cdot 3(CH_2Cl_2)=C_{71}H_{70}Cl_6N_{16}O_{14}S_4$, colorless cube, M_r=1712.413, monoclinic $P2_1$, a=10.5360 (4) Å, b=24.3470 (8) Å, c=15.9700 (8) Å, α=90.00°, β=95.1770 (10)°, γ=90.00°, V=4079.9 (3) Å^3, Z=2, ρ_{calcd}=1.394 mg/m^3, μ=0.384/mm, T=298 K, 14144 measured reflections, 14122 independent reflections, 1000

parameters, GOF=1.062, and $R1$ ($wR2$)=0.0639 (0.1764). The measurements were carried out by a Mac Science (Bruker Nonius) dip image plate diffractometer, using graphite-monochromated Mo K_α radiation (λ=0.71073 Å). The crystal structure was solved by the direct method with SIR-97. Refinement was performed by full-matrix least-squares refinement on F^2 with SHELXL-97. CCDC 627198 contains supplementary crystallographic data to that presented in this paper. These data can be obtained free of charge from Cambridge Crystallographic Data Centre *via* www.ccdc.cam.ac.uk/data_request/cif.

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