

Kettapeptin: Isolation, Structure Elucidation and Activity of a New Hexadepsipeptide Antibiotic from a Terrestrial *Streptomyces* sp.

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Abstract The ethyl acetate extract of the *Streptomyces* sp. isolate GW99/1572 exhibited significant biological activity against Gram-positive bacteria and delivered kettapeptin (**1**), a new hexadepsipeptide antibiotic of the azinothricin type. The structure was elucidated by various 1D and 2D NMR techniques, mass spectrometry and by comparison of the NMR data with those of closely related antibiotics. The absolute configuration of the compound was derived by crystal structure analysis and by comparison with the optical rotation data of related compounds.

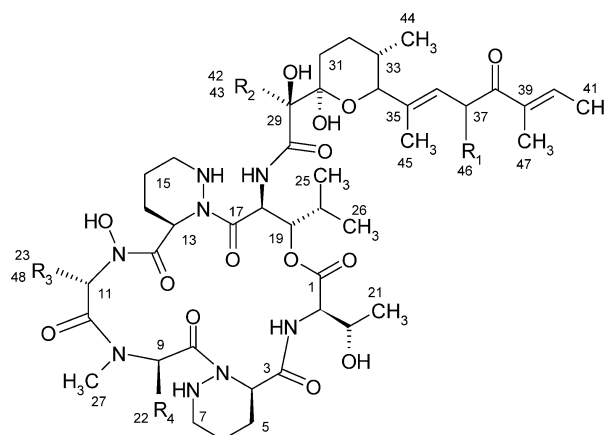
Keywords kettapeptin, peptide lactone, antibiotic, cytotoxic, azinothricin

Introduction

Cyclic hexadepsipeptides of the azinothricin type are characterised by a 19-membered cyclodepsipeptide ring composed of 6 unusual amino acids and an acyl side chain connected through an amide bond. The first member of this class, azinothricin (**2**) was reported from *Streptomyces* X-14950 in 1986 [1]. These antibiotics exhibit strong

antitumor and antibacterial activity against Gram-positive bacteria [2~4]. Many of them show activity against peptic and duodenal ulcer [5]. Additionally, they are known to promote wound healing [6], and have antiinflammatory activity [7].

In our screening program of streptomycetes for new novel antibiotics, the ethyl acetate extract of the strain *Streptomyces* sp. isolate GW99/1572 exhibited biological activity against the bacteria *Bacillus subtilis*, *Escherichia coli*, and *Streptomyces viridochromogenes*, the fungi *Candida albicans* and *Mucor miehei* and the micro-alga *Scenedesmus subspicatus*. Responsible for the antifungal



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- 1: $R^1 = R^4 = \text{CH}_3$; $R^2 = \text{CH}_2\text{CH}_3$; $R^3 = \text{CH}_2\text{OCH}_3$
- 2: $R^1 = R^2 = \text{CH}_2\text{CH}_3$; $R^3 = \text{CH}_2\text{OCH}_3$; $R^4 = \text{CH}_3$
- 3: $R^1 = R^3 = R^4 = \text{CH}_3$; $R^2 = \text{CH}_2\text{CH}_3$
- 4: $R^1 = R^2 = R^3 = \text{CH}_3$; $R^4 = \text{CH}_2\text{CH}(\text{CH}_3)_2$
- 5: $R^1 = R^2 = \text{CH}_3$; $R^3 = \text{CH}_2\text{OCH}_3$; $R^4 = \text{CH}_2\text{CH}(\text{CH}_3)_2$

activity was a complex mixture (ESI MS) of yellow polar antibiotics that were identified as carbonyl-conjugated polyene macrolides by UV/VIS, NMR data, and the brown colour reaction with concentrated sulphuric acid; these compounds were not further characterised. In addition, a colourless weakly UV absorbing TLC band was detected which gave a violet colouration with anisaldehyde/sulphuric acid. In a bio-autogram, the band was shown to be responsible for the anti-microbial activity mentioned above. Work-up of the extract guided by biological activity and chemical screening resulted in the isolation of a new hexadepsipeptide antibiotic that we name kettapeptin (**1**). In this paper, we report on the taxonomy of the producing strain and the isolation, structure elucidation and biological activity of the new antibiotic.

Results and Discussion

The terrestrial *Streptomyces* sp. isolate GW99/1572 was cultivated in M₂ medium using our standard conditions [8]. The crude extract resulting from ethyl acetate extraction was subjected to column chromatography on silica gel using a CH₂Cl₂/MeOH gradient and separated into two fractions. Further purification of the more polar fraction using preparative TLC and Sephadex LH-20 afforded kettapeptin (**1**) in a yield of 4 mg per liter of fermentation.

Kettapeptin (**1**) was obtained as a colourless solid. The colour with the chlorine/tolidine reaction and the negative ninhydrin test indicated it to be an *N*-terminal blocked peptide. The positive and negative ESI spectra showed *quasi*-molecular ion peaks at *m/z* 1029 ([M+Na]⁺) and 1005 ([M-H]⁻), respectively. This was consistent with the molecular weight of 1006 (C₄₈H₇₈N₈O₁₅ by HRMS). The IR spectrum contained an ester band at ν 1744 cm⁻¹ and amide bands at ν 1667, 1650 and 1636 cm⁻¹. The complex ¹H NMR spectrum delivered eight signals of acidic protons and at least six α -methine protons of amino acids between δ 6.13 and 4.54, characteristic of peptides. It also showed two olefinic proton signals at δ 6.74 and 5.63 as indicated by HSQC correlations. In addition to many other methine and methylene proton signals, the spectrum depicted twelve methyl signals, an *O*- and an *N*-methyl singlet, an olefinic methyl doublet, two olefinic methyl singlets, and five doublets and a triplet for methyl groups connected to *sp*³ carbon atoms. With the aid of ¹H-¹H COSY couplings, an ethyl and an isopropyl residue were identified. The search with these spectroscopic data in AntiBase [9] did not reveal a structure that matched the NMR data, the molecular weight and the empirical formula of **1**. However, the search uncovered hexadepsipeptides with similar analytical data:

azinothricin [**1**] (**2**), A83586C [10] (**3**), GE3 (**4**) [2] and citropeptin [11] (**5**). The similarity of the NMR data with those of **2**~**5** indicated that the isolated compound might be a related cyclopeptidelactone.

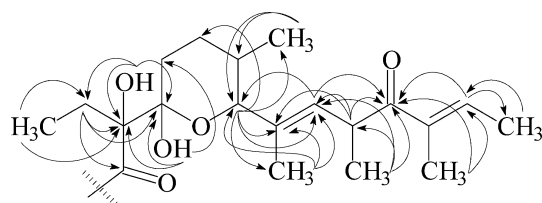
The ¹³C and APT NMR spectra of kettapeptin (**1**) depicted 48 carbon signals. It contained, in addition to a signal at δ 203.0 of a conjugated ketone, seven amide carbonyl signals between δ 175.3~169.5. In the *sp*² carbon region, two quaternary and two methine carbon signals were detected. Signals for a quaternary acetal carbon at δ 99.6 and a quaternary carbon atom bearing oxygen at δ 80.1 were visible in the spectrum. Ten methylene carbon signals were also observed: one with an oxygen atom, two bearing nitrogen atoms and the residuals connected only to *sp*³ carbon atoms. The spectrum also contained six α -methine carbons of amino acids between δ 56.2~47.7, three signals for CH groups connected to oxygen and three more methine signals at δ 38.2, 32.5 and 29.2. In addition, twelve methyl signals included one *O*-methyl, one *N*-methyl and nine of *C*-methyl groups. Careful interpretation of the ¹H and ¹³C NMR data and ¹H-¹H COSY, HSQC and HMBC couplings resulted in an acyl substructure (Fig. 1) and a cyclic peptide backbone (Fig. 2). The HMBC coupling of 18-CH and 18-NH of the peptide substructure (Fig. 2) to the carbonyl carbon of the acyl side chain (Fig. 2) connected both fragments to give the final planer structure of kettapeptin (**1**).

NOE effects between 45-H₃ and 46-H₃ as well as 41-H₃ and 47-H₃ indicated a *trans,trans*-configuration of the double bonds in the side chain. The coupling constant of *J*=10 Hz between 33-H (δ 1.48) and 34-H (δ 3.98) indicated these protons to be in an axial position. The NOE couplings of 30-OH with 42-H₂, 43-H₃ and 34-H and of 34-H with 44-H₃ and the similarity of the ¹H and ¹³C data of the respective proton and carbon atoms with those of **2**~**5** suggested a relative configuration of the tetrahydropyranyl part as in **2**~**5**. The ¹H chemical shift of 37-H (δ 4.09) and 46-H₃ (δ 1.14) and the ¹³C data of C-37 (δ 38.2) and C-46 (δ 19.5) of **1** were very similar to those of GE3 (**4**; 37-H: δ 4.06; 46-H₃: δ 1.12; C-37: δ 38.5; C-46: δ 19.2), A83586C (**3**; 37-H: δ 4.09; 46-H₃: δ 1.12; C-37: δ 38.3; C-46: δ 19.6) and citropeptin (**5**; 37-H: δ 4.07; 46-H₃: δ 1.12; C-37: δ 38.0; C-46: δ 18.8) and indicated that the acyl substructure possessed the same relative configuration, related conformations anticipated.

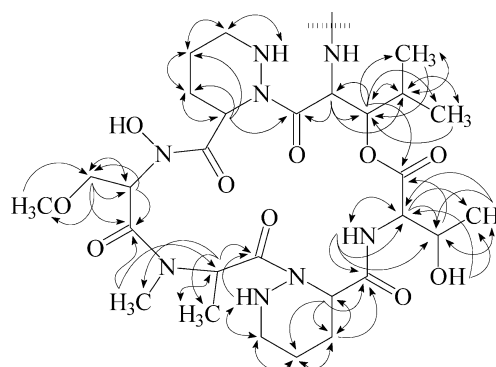
As kettapeptin (**1**) crystallized easily from acetone, a crystal structure analysis was performed (**1**) which indeed confirmed our assumption: The relative configuration of **1** derived from the NMR data (Table 1) and the crystal analysis (Fig. 3) is identical to those of **2**~**5**. As also the specific rotation ($[\alpha]_D^{20}$ =+111.2°) of **1** is very similar to

Table 1 ^1H (600 MHz) and ^{13}C (150 MHz) NMR data of kettapeptin (**1**) in CDCl_3 (δ ; J in Hz)

C No.	^{13}C NMR	^1H NMR	C No.	^{13}C NMR	^1H NMR
1	170.2 s	—	24	29.2 d	1.68 (m)
2	56.2 d	4.54 (d, 8.6)	25	19.3 q	0.72 (d, 7.1)
3	169.5 s	—	26	14.6 q	0.83 (d, 6.8)
4	52.4 d	5.20 (dd, 5.6, 1.5)	27	29.4 q	3.06 (s)
5	24.4 t	2.58 (m), 1.72 (m)	28	175.3 s	—
6	21.3 t	1.64 (m), 1.54 (m)	29	80.1 s	—
7	48.0 t	3.30 (d, 13.2), 2.58 (m)	29-OH	—	2.95 (s)
7-NH	—	3.87 (m)	30	99.6 s	—
8	173.1 s	—	30-OH	—	6.29 (s)
9	47.7 d	6.13 (q, 7.0)	31	28.3 t	1.72 (m)
10	171.0 s	—	32	27.2 t	1.52 (m)
11	53.8 d	5.34 (t, 7.1)	33	32.5 d	1.48 (m)
11-NOH	—	9.96 (s)	34	82.2 d	3.98 (d, 10.0)
12	172.8 s	—	35	132.8 s	—
13	51.3 d	4.92 (m)	36	129.3 d	5.63 (dd, 9.1, 0.7)
14	24.0 t	2.26 (d, 13.4), 1.88 (m)	37	38.2 d	4.09 (dq, 8.9, 6.9)
15	21.1 t	1.54 (m), 1.48 (m)	38	203.0 s	—
16	45.6 t	3.17 (d, 12.8), 2.98 (m)	39	137.5 s	—
16-NH	—	4.44 (dd, 12.2, 1.6)	40	136.8 d	6.74 (q, 6.9)
17	170.8 s	—	41	14.9 q	1.86 (m)
18	54.6 d	4.92 (m)	42	25.9 t	2.04 (m), 1.66 (m)
18-NH	—	8.24 (d, 10.7)	43	8.4 q	0.86 (t, 7.6)
19	78.6 d	5.42 (dd, 10.7, 2.1)	44	17.6 q	0.71 (d, 6.2)
20	64.7 d	4.80 (q, 6.5)	45	12.0 q	1.59 (s)
20-OH	—	4.37 (s)	46	19.5 q	1.14 (q, 6.8)
21	18.9 q	1.07 (d, 6.5)	47	11.4 q	1.78 (s)
22	13.1 q	1.26 (d, 7.0)	48	59.2 q	3.37 (s)
23	68.5 t	3.87 (m), 3.76 (dd, 10.0, 6.7)			

**Fig. 1** Structure of the acyl rest of kettapeptin (**1**) derived by HMBC (\rightarrow) correlations.

that of azinothricin (**2**, $[\alpha]_{\text{D}}^{25} = +117.65^\circ$) [1], A83586C (**3**, $[\alpha]_{\text{D}}^{25} = +116.1^\circ$) [10], GE3 (**4**, $[\alpha]_{\text{D}}^{25} = +111.5^\circ$) [2], and citropeptin (**5**, $[\alpha]_{\text{D}}^{25} = +113.0^\circ$) [11], also the absolute configuration of **1** should be identical with that of **2**~**5**. The peptide lactone part of kettapeptin (**1**) is identical with that of azinothricin (**2**) and very similar to those of A83586C (**3**), GE3 (**4**) and citropeptin (**5**). Kettapeptin (**1**) is the 37-*nor*-derivative of azinothricin (**2**).

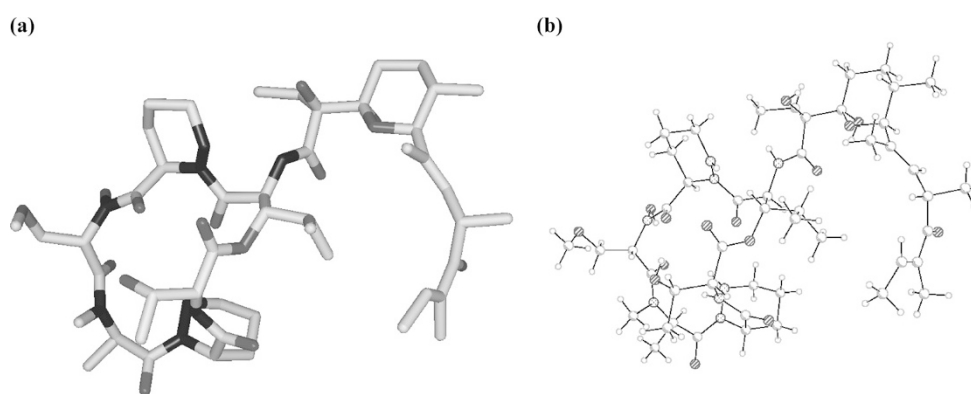
**Fig. 2** Structure of the peptide chain of kettapeptin (**1**) derived by ^1H - ^1H COSY (\leftrightarrow) and HMBC (\rightarrow) correlations.

Biological Activity

Antibacterial, antifungal and antialgal activities were *semiquantitatively* determined using the agar diffusion method with 9 mm paper discs and 0.1~ μg of kettapeptin

Table 2 Physicochemical properties of kettapeptin (**1**)

Appearance	Colourless crystals (from acetone)
R _f (CH ₂ Cl ₂ /5% MeOH)	0.35
Molecular formula	C ₄₈ H ₇₈ N ₈ O ₁₅
(+)-ESI MS: <i>m/z</i> (%)	1029 ([M+Na] ⁺ , 100), 1051 ([M+2Na-H] ⁺ , 8)
(-)-ESI MS: <i>m/z</i> (%)	1027 ([M+Na-2H] ⁻ , 20), 1005 ([M-H] ⁻ , 100)
ESI HRMS	1006.5617 (calcd. 1006.558664)
IR (KBr): $\nu_{\text{cm}^{-1}}$	3422, 2955, 2938, 1744, 1667, 1650, 1636, 1504, 1458, 1394, 1352, 1318, 1260, 1208, 1148, 1118, 1069, 998, 912, 869, 818, 728, 686
$[\alpha]_{\text{D}}^{20}$ (c 1.0, CHCl ₃)	+111.2

**Fig. 3** Crystal structure of kettapeptin (**1**) (a) without and (b) with the hydrogen atoms**Table 3** Antibacterial activities of kettapeptin (**1**) and bacitracin A in agar diffusion test (i.d. of inhibition zones, mm)

Compounds	Amount ($\mu\text{g}/\text{disk}$)	BS ^a	SV ^b	SA ^c	EC ^d
Kettapeptin (1)	0.1	0			
	1	10			
	5	13	16	20	12
	10	14	16	17	13
	20	16	16	16	13
Bacitracin A	5	0	0	0	0
	10	11	0	11	0
	20	14	0	12	11

^a *Bacillus subtilis*, ^b *Streptomyces viridochromogenes* (Tü 57), ^c *Staphylococcus aureus*, ^d *Escherichia coli*.

(**1**)/disk using bacitracin A as a standard. Kettapeptin (**1**) exhibited activity against *Bacillus subtilis*, *Streptomyces viridochromogenes* (Tü 57), *Staphylococcus aureus* and *Escherichia coli*, which proved to be much better than that of bacitracin A (Table 3); the MIC value against *Bacillus*

subtilis was determined as 3.75 $\mu\text{g}/\text{ml}$. The compound showed no activity against *Candida albicans*, *Mucor miehei*, and the microalgae *Chlorella vulgaris*, *Chlorella sorokiniana*, and *Scenedesmus subspicatus*.

In addition to the antibacterial activity, kettapeptin (**1**)

was found to be highly active against human cancer cell lines LXFA 629L and LXFL 529L (lung cancer), MAXF 401NL (breast tumor), MEXF 462NL (melanoma), RXF 944L (kidney tumor) and UXF 1138L (uterus tumor) [12] with IC_{70} value of $<0.6 \mu\text{g/ml}$.

Experimental

Materials and Methods

NMR spectra were measured on a Varian Inova 600 (600 MHz) spectrometer. ESI-MS was recorded on a Quattro Triple Quadrupole Mass Spectrometer, Finnigan TSQ 7000 with nano-ESI-API-ion source. ESI HRMS were measured on Micromass LCT mass spectrometer coupled with a HP 1100 HPLC with a Diode Array Detector. Reserpin (MW=608) and leucin-enkephalin (MW=555) were used as standards in positive and negative mode. IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrometer as KBr pellets. UV-VIS spectra were recorded on a Perkin-Elmer Lambda 15 UV/VIS spectrometer. Flash chromatography was carried out on silica gel (230~400 mesh). Thin layer chromatography (TLC) were performed on Polygram SIL G/UV₂₅₄ (Macherey-Nagel & Co.). Rf values were measured on Polygram SIL G/UV₂₅₄ (Macherey-Nagel & Co.). Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia). The X-ray structure analysis was performed at 100 K using a Bruker rotating anode X-ray source operating at Cu $K\alpha$ radiation (1.5418 Å wavelength), equipped with osmic focussing mirrors, Bruker platform goniometer and a Bruker SMART 6000 CCD detector.

Taxonomy

Actinomycete strain GW99/1572 was obtained from the strain collection of Labor Grün-Wollny, Giessen, Germany, where it is maintained. The bacterium was Gram-positive, non-acid fast, grew aerobically, and differentiated into substrate and aerial mycelium. The aerial hyphae were highly branched with long straight to *flexous* spore chains. Neither aerial nor substrate mycelium showed fragmentation.

The colour of the aerial mycelium was firstly light grey and turned later into green on yeast extract - malt, oatmeal and soil extract agar. The substrate mycelium was light brown on these media. A light orange diffusible pigment was formed on yeast extract - malt extract agar and on soil extract agar. Melanin pigments were not produced on tyrosine agar slants.

The diaminopimelic acid isomer and the sugar composition of the whole cell hydrolysate indicated that the

strain had cell walls of type I and belongs to the genus *Streptomyces*.

Fermentation, Extraction and Isolation

With a well grown agar culture of the terrestrial *Streptomyces* sp. isolate GW99/1572, one hundred 1 litre-Erlenmeyer flasks each containing 250 ml of M₂ medium (10 g malt extract, 4 g yeast extract and 4 g glucose in 1 l of tap water was set to pH 7.8 with 2 N NaOH and sterilized for 30 minutes at 121°C) were inoculated and incubated for 4 days at 28°C on a linear shaker (110 rpm). The culture broth was mixed with *ca.* 1 kg of diatomaceous earth and filtered through a press filter to separate mycelium and water phase. The mycelial cake and the filtrate were separately extracted each three times with ethyl acetate (*ca.* 2 litres each). Since the chemical compositions of both organic phases were similar, they were combined and concentrated under reduced pressure to yield 4.7 g of a yellow oily crude extract.

The crude extract was then subjected to column chromatography on silica gel (3×200 cm, 200 g) with a dichloromethane - methanol gradient and separated into two fractions (I and II) using TLC to monitor the separation. The first fraction contained a complex mixture of hydroxylated polyenemacrolides and was not further analysed. The second fraction exhibited the antibacterial activity and contained a colourless band on TLC, which turned to light violet with anisaldehyde/sulphuric acid. Purification of the fraction by preparative TLC (20×20 cm, CH₂Cl₂/7% MeOH) followed by final purification on Sephadex LH-20 (CH₂Cl₂/60% MeOH) delivered 110 mg of kettapeptin (**1**) as a colourless solid.

Crystal Structure Analysis

Kettapeptin crystallized from acetone as needles in the orthorhombic space group P2₁2₁2₁ with a single molecule in the asymmetric unit. For data collection, the crystal was shock frozen in a cold nitrogen stream using perfluoropolyether oil as cryoprotectant.

The data reduction was performed using *SAINTE* (Version 6.05, Bruker AXS 1997–1999) and the data were corrected *semiempirically* for absorption and other effects with SADABS [13]. The phase problem was solved by conventional direct methods using *SHELXS* [14] and the model was refined against F² on all data by full-matrix least-squares with *SHELXL* [15]. All non-hydrogen atoms were refined anisotropically. All hydrogen atoms were included at geometrically calculated positions.

Crystal data: C₄₈H₇₈N₈O₁₅ + 1 CH₃COCH₃, crystal size: 0.2 mm×0.1 mm×0.05 mm, space group P2₁2₁2₁, unit cell a=9.780 (2) Å, b=20.355 (4) Å, c=28.635 (6) Å, $\alpha=\beta=\gamma$

=90°, non-hydrogen atomic volume=18.3 Å³, ρ_{calc} =1.226 g/cm⁻³, 26627 reflections were measured, 3896 unique reflections, resolution=28.49–0.95 Å, completeness (%)=97.1, R (int) (%)=0.0827, I/σ =12.36, ($R_{\text{int}} = \Sigma|I - \langle I \rangle|/\Sigma I$). The refinement converged to $R_1 = \Sigma||F_o| - |F_c||/\Sigma|F_o| = 0.0576$ for 3142 reflections $F_o > 4\sigma$ and 0.0782 for all 3896 data, $wR_2 = [\Sigma w(F_o^2 - F_c^2)/\Sigma w(F_o^2)]^{1/2} = 0.1579$ for 3896 data and 2/676 parameters, goodness of fit is 1.034. The structure has been submitted to Cambridge Structure data base with CCDC number 603484.

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