# Rakicidin C, A New Cyclic Depsipeptide from *Streptomyces* sp.

**Jin-Feng Hu,[a] Dirk Wunderlich,[a] Isabel Sattler,[a] Xiao-Zhang Feng,[b] Susanne Grabley,[a] and Ralf Thiericke*[a]**

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Chemical screening of extracts of Actinomycetes strains has led to the detection, isolation, and structure elucidation of the new cyclic depsipeptide rakicidin C (**1**). The secondary metabolite from *Streptomyces* sp. (strain GT 61042) is built-up from glutamine, *N*-methyl glycine, 4-amino-(2*E*,4)-pentadienoic acid, and 3-hydroxy-2,4,6,8-tetramethylnonanoic acid.

## Introduction

In the search for new secondary metabolites, application of our chemical screening routine to the culture broths of various microorganisms has resulted in the discovery of a number of new compounds.[1] In continuation of this screening approach on different *Streptomyces* strains,[2] we have now investigated the secondary metabolites of the actinomycete isolate *Streptomyces* sp. (strain GT 61042). The new fifteen-membered cyclic depsipeptide rakicidin C (**1**) thus discovered is structurally related to the known cytotoxic depsipeptides rakicidins A (**2**) and B (**3**),[3] which were obtained from the culture broth of a *Micromonospora* species (Scheme 1).

For screening purposes, the cultivation of *Streptomyces* sp. (strain GT 61042) was carried out in 300 mL Erlenmeyer flasks on a rotary shaker using a culture medium containing casein peptone (medium 1, 100 mL). The secondary metabolite profile of strain GT 61042 was examined by TLC analysis, which led us to investigate this strain in more detail (Table 1).

In order to isolate the metabolites detected by the chemical screening method, *Streptomyces* sp. (strain GT 61042) was cultivated in a 300 L fermentor using medium 1 at 28 °C for 6 days. The culture filtrate was then adsorbed on Amberchrom CG/161 M and eluted with an $H_2O$/MeOH gradient. The desired metabolite was found to be enriched in the fraction containing 10% methanol.

## Results and Discussion

The molecular weight of **1** and its chemical formula of $C_{26}H_{42}N_4O_6$ were readily inferred from the positive mode high-resolution electrospray ionization mass spectrum (HR-ESIMS), which showed the $[M + H]^+$ ion peak ($m/z$ = 507.3182; $C_{26}H_{43}N_4O_6$ requires 507.3172). Supporting evidence was obtained from low-resolution electrospray ionization mass spectra (positive and negative mode), which displayed peaks at $m/z$ = 507 ($[M + H]^+$), 529 ($[M + Na]^+$), 1035 ($[2M + Na]^+$), and 505 ($[M - H]^-$). The $^{13}C$ and DEPT NMR spectra showed signals due to 26 carbons, which could be classified as six methyl, six methylene, eight methine, one $sp^2$ quaternary, and five carbonyl carbons. The $^1H$ NMR spectrum recorded in $CD_3OD$ solution showed signals due to 38 protons (Table 2), whereas that recorded in [$D_6$]DMSO featured four additional proton signals (δ = 7.36, 7.29, 7.26, and 6.85) suggesting the presence of exchangeable NH groups. In the lowfield region of the $^1H$ NMR spectrum ($CD_3OD$), four olefinic proton signals were seen at δ = 5.45 and 5.51 (each s, 1 H, *exo*-methylene, 13a-H and 13b-H), 6.15 (d, 1 H, 10-H), and 7.10 (d, 1 H, 11-H) [the (*E*)-configuration was confirmed by the coupling constant of 14.9 Hz]. In the high-field region of the $^1H$ NMR spectrum ($CD_3OD$), signals due to a branched aliphatic side chain were observed. In addition, the following proton signals were also observed: three methine signals at δ = 4.55 (2-H), 2.85 (15-H), and 5.35 (16-H), and three non-equivalent methylene signals at δ = 1.95/2.15 (3-$H_2$), 2.25 (4-$H_2$), and 3.95/4.45 (7-$H_2$). A singlet due to one methyl group was seen at δ = 3.15 (8-$H_3$). On the basis of this shift, together with the $^{13}C$ NMR chemical shift of δ = 37.58 (C-8), this methyl group could be assigned as being linked to an amide nitrogen.

From careful examination of the $^1H$-$^1H$ COSY NMR spectrum, the presence of the molecular fragments shown in Figure 1 was established. The connectivity of proton and carbon atoms was confirmed by a heteronuclear single quantum coherence (HSQC) NMR experiment. On the basis of these findings, $^{13}C$-$^1H$ long-range couplings, $^2J$ and $^3J$, were measured in a heteronuclear multiple bond correlation (HMBC) NMR experiment (Figure 2), which were indicative of the cyclic structure shown in Scheme 1. By detailed analysis of the $^1H$ and $^{13}C$ NMR (Table 2), $^1H$-$^1H$ COSY, HSQC, and HMBC spectral data, it was established that **1** is assembled from three amino acids and a 3-hydroxy fatty

[a] Hans-Knöll-Institute for Natural Products Research, Beutenbergstraße 11, 07745 Jena, Germany
E-mail: thierick@pmail.hki-jena.de
[b] Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, No. 1, Xian Nong Tan Street, Beijing 100050, P. R. China

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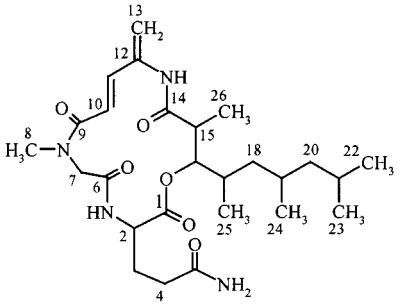
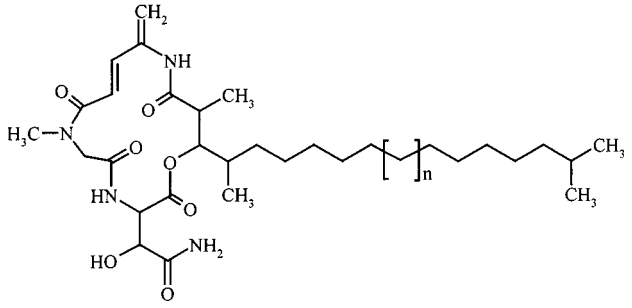
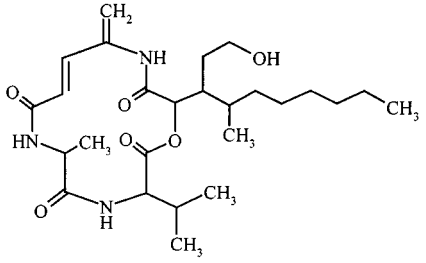


Rakicidin C (**1**)



Rakicidin A (**2**): n = 1
Rakicidin B (**3**): n = 2



Vinylamycin (**4**)

Scheme 1. The structures of rakicidin C (**1**), rakicidins A (**2**) and B (**3**),[3] and vinylamycin (**4**)[4]

Table 1. $R_f$ values and color reactions of rakicidin C (**1**) on silica gel TLC plates

| | **1** |
|---|---|
| *Solvent system* | |
| $CHCl_3$/MeOH (9:1) | 0.14 |
| $CHCl_3$/MeOH (5:1) | 0.45 |
| 1-Butanol/acetic acid/water (4:1:5, upper phase) | 0.41 |
| *Staining reagent*[a] | |
| Anisaldehyde/$H_2SO_4$ reagent | brown |
| Ehrlich's reagent[b] | white |
| Orcinol reagent | brown |
| Blue tetrazolium reagent | light purple |
| Naphthoresorcinol/$H_2SO_4$ reagent | brown-black |
| 5% $FeCl_3-H_2O$ (2.5 g $FeCl_3$, 48 mL $H_2O$, 2 mL 25% HCl) | grey-black[c] |

[a] After spraying, the TLC plates were heated at 120 °C for 5 min. – [b] On a yellow background. – [c] This color can be easily observed, but disappears after ca. 0.5 h.

Table 2. $^1H$ (300 MHz, δ values, $CD_3OD$) and $^{13}C$ (75 MHz) NMR data of rakicidin C (**1**)

| Position | $^1H$[a] | $^{13}C$ (DEPT)[a] |
|---|---|---|
| 1 | | 170.98 (C) |
| 2 | 4.55 (1 H, dd, *J* = 4.9, 10.1) | 53.84 (CH) |
| 3 | 1.95 (1 H, m), 2.15 (1 H, m) | 30.86 ($CH_2$) |
| 4 | 2.25 (2 H, t, *J* = 7.3) | 32.95 ($CH_2$) |
| 5 | | 177.23 (C) |
| 6 | | 170.17 (C) |
| 7 | 3.95 (1 H, d, *J* = 18.0), 4.45 (1 H, d, *J* = 18.0) | 53.97 ($CH_2$) |
| 8 | 3.15 (3 H, s) | 37.58 ($CH_3$) |
| 9 | | 168.79 (C) |
| 10 | 6.15 (1 H, d, *J* = 14.9) | 118.95 (CH) |
| 11 | 7.10 (1 H, d, *J* = 14.9) | 141.25 (CH) |
| 12 | | 139.40 (C) |
| 13a/b | 5.45 (1 H, s), 5.51 (1H, s) | 120.25 ($CH_2$) |
| 14 | | 175.64 (C) |
| 15 | 2.85 (1 H, dq, *J* = 7.0, 10.2) | 44.23 (CH) |
| 16 | 5.35 (1 H, dd, *J* = 1.7, 10.2) | 78.24 (CH) |
| 17 | 2.01 (1 H, m) | 32.12 (CH) |
| 18 | 0.95 (1 H, m), 1.24 (1H, m) | 43.47 ($CH_2$) |
| 19 | 1.78 (1 H, m) | 28.39 (CH) |
| 20 | 1.00 (2 H, m) | 48.32 ($CH_2$) |
| 21 | 1.63 (1 H, m) | 26.39 (CH) |
| 22 | 0.88 (3 H, d, *J* = 6.5) | 24.34 ($CH_3$) |
| 23 | 0.86 (3 H, d, *J* = 6.6) | 22.38 ($CH_3$) |
| 24 | 0.87 (3 H, d, *J* = 6.6) | 20.15 ($CH_3$) |
| 25 | 1.02 (3 H, d, *J* = 6.8) | 14.10 ($CH_3$) |
| 26 | 1.21 (3 H, d, *J* = 7.0) | 15.83 ($CH_3$) |

[a] Assignments were made by a combination of 1D- ($^1H$, $^{13}C$, DEPT) and 2D NMR ($^1H$-$^1H$ COSY, HSQC, HMBC) experiments; *J* values in Hz.

acid: (i) glutamine, (ii) *N*-methyl glycine, (iii) 4-amino-(2*E*,4)-pentadienoic acid, and (iv) 3-hydroxy-2,4,6,8-tetramethylnonanoic acid.

In general, MS/MS has proved to be a powerful method for elucidating amino acid sequences in peptides. However, the MS/MS trace of the cyclic depsipeptide **1** was ambiguous. The successful long-range HMBC experiments were helpful in assigning the linkage points of the peptide and ester bonds (Figure 2). Thus, it was established that the glutamine, *N*-methyl glycine, 4-amino-(2*E*,4)-pentadienoic acid, and 3-hydroxy fatty acid are linked through amide bonds. The glutamine and the 3-hydroxy fatty acid form an ester bond (Figure 2). The determination of the stereochemistry of rakicidin C (**1**) is under investigation.

Recently, the depsipeptides rakacidins A and B (**2** and **3**)[3] as well as vinylamycin (**4**)[4] have been described, which have an exocyclic double bond moiety like that in **1**

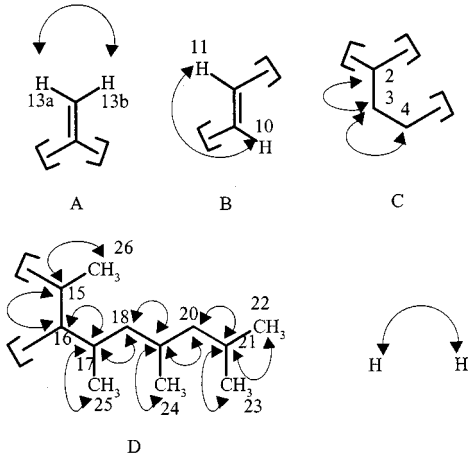



Figure 1. Fragments of rakicidin C (**1**) deduced from a $^1$H-$^1$H COSY NMR experiment

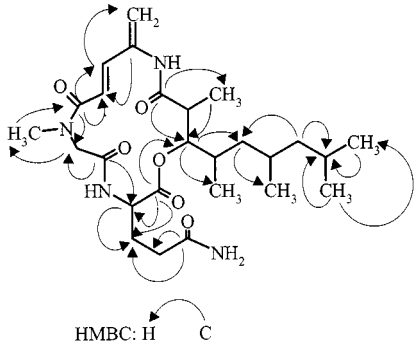



Figure 2. Key $^nJ_{CH}$ ($n$ = 2 or 3) correlations in rakicidin C (**1**) as deduced from an HMBC experiment

(Scheme 1). Rakacidins A (**2**) and B (**3**) are fifteen-membered ring depsipeptides with a 3-hydroxy fatty acid building block (Scheme 1). The new rakacidin C (**1**) also contains a fifteen-membered ring with an identical constitution as that in the known rakacidins A (**2**) and B (**3**). Rakacidin C (**1**) differs from rakacidins A (**2**) and B (**3**) in two respects, having: (i) a different 3-hydroxy fatty acid moiety, and (ii) glutamine in place of the 3-hydroxyasparagine in **2** and **3**.[3] Vinylamycin (**4**), obtained from *Streptomyces* sp. MI982-63F1, is a fourteen-membered ring depsipeptide with a 2-hydroxy fatty acid as a building block.[4]

Rakicidin C (**1**) has been tested against gram positive and gram negative strains, as well as with regard to its cytotoxicity towards various cell lines, but was found to be inactive.

## Experimental Section

**General Methods:** For aspects of the instrumentation and general methods, see the previous paper.[2] − 1D ($^1$H, $^{13}$C, and DEPT) and 2D ($^1$H-$^1$H COSY, HSQC, HMBC, and NOESY) NMR spectra were recorded on Bruker DPX 300 and Bruker DRX 500 spectrometers, respectively. − IR spectra were recorded on a Bruker FT-IR (IFS 55) spectrometer. − UV/vis spectra were recorded on a Cary 1 Bio UV/vis spectrophotometer (Varian). − ESI mass spectra were obtained on VG Quattro Micromass, MAT95 Thermoquest, and AMD Intectra 402/2 mass spectrometers.

**Culture Material:** Pure cultures of strain GT 61042, obtained by standard isolation procedures for *Streptomycetes* strains from a soil sample, were grown on medium 1 [glycerol 30 g/L, casein peptone 2.0 g/L, $K_2HPO_4$ 1.9 g/L, $Na_2SO_4$ 1.6 g/L, $MgSO_4 \cdot 7\,H_2O$ 0.5 g/L, trace element solution ($CaCl_2 \cdot 2\,H_2O$ 3.0 g, $FeC_6H_5O_7$ 1.0 g, $MnSO_4$ 0.2 g, $ZnCl_2$ 0.1 g, $CuSO_4 \cdot 5\,H_2O$ 0.025 g, $Na_2B_4O_7 \cdot 10\,H_2O$ 0.02 g, $Na_2MoO_4 \cdot 2\,H_2O$ 0.01 g, and $CoCl_2$ 0.004 g per dm$^3$ deionized water) 5 mL/L] for 4 days at 28 °C. The pure strain was stored in 50% glycerol at −20 °C.

**Fermentation:** The glycerol-containing storage mixture (2 mL) was used to inoculate a 300 mL Erlenmeyer flask containing 100 mL of medium 1. Cultivation was carried out by placing the flask on a rotary shaker (180 rpm) at 28 °C for 6 days and then the solution was subjected to initial TLC analysis as part of the routine screening procedure. Details of the chemical screening method (cultivation, adsorption of the culture filtrate, concentration steps, and the conditions for TLC analysis) have been reported previously.[5,6] Four-day-old cultures prepared in the same manner were used to inoculate a fermentor (300 L working volume, inoculation volume 5%, 400 rpm, 28 °C, aeration 5 L/min) containing medium 1. It was found that foaming could be decreased by adding PPG (polypropylene glycol).

**Isolation and Purification:** After harvesting, the culture filtrate (160 L) was filtered and adsorbed onto Amberchrom CG/161 M (6 L of resin) by means of MPLC and eluted with an $H_2O$/MeOH gradient (0−2.0 min: 30% MeOH; 2.0−26.0 min: 30%−80% MeOH; 26.0−26.1 min: 80−100% MeOH; 26.1−38.0 min: 100% MeOH; 38.0−39.0 min: 100%−10% MeOH; 39.0−45.0 min 10% MeOH). The eluates were concentrated to leave aqueous residues, which were then lyophilized to yield seven fractions. The crude material obtained from fraction 6 (10% MeOH, between 39.0 and 45.0 min, 300 mg, monitored by TLC analysis) was redissolved in MeOH (500 mL) by stirring at room temperature, the resulting solution was filtered, and the filtrate was concentrated to dryness under reduced pressure to yield a black residue (56 mg). This material was chromatographed on silica gel (column: 2.2 × 32 cm; eluent: $CHCl_3$/MeOH, 5:1) to furnish pure rakicidin C (**1**, 0.2 mg/L) as a white powder.

**Rakicidin C (1):** $[\alpha]_D^{25}$ = +90.9 ($c$ = 0.237, DMSO). − ESI-MS (negative mode): $m/z$ = 505 [M − H]$^-$. − IR (KBr): $\tilde{\nu}_{max}$ = 3360 cm$^{-1}$ (br), 2957, 2929, 2861, 1717, 1653 (br), 1533, 1456, 1274, 1048. − UV (MeOH): $\lambda_{max}$ (log ε) = 201 nm (3.73), 243 (1.71). − $^1$H and $^{13}$C NMR (in $CD_3OD$): see Table 2.

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