# Rakicidins, New Cytotoxic Lipopeptides from *Micromonospora* sp. Fermentation, Isolation and Characterization

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The new cytotoxic agents rakicidins A and B were isolated from cultured broth of a *Micromonospora* sp. Spectroscopic and amino acid analysis has shown that rakicidin A is a new cyclic lipopeptide, consisting of 4-amino-penta-2,4-dienoic acid, 3-hydroxy-2,4,16-trimethyl-heptadecanoic acid, sarcosine, and 3-hydroxyasparagine. Rakicidin B differs by one methylene group in the lipid side chain. These compounds exhibited cytotoxicity against the M109 cell line.

In our continuing search for new antitumor agents from microorganisms, an isolate of *Micromonospora* was selected for further evaluation. This research led to the discovery of two new cytotoxic lipopeptides, rakicidins A and B (Fig. 1). This paper describes the fermentation, biological evaluation, purification, physico-chemical characterization, and structure elucidation of these compounds.

## Fermentation

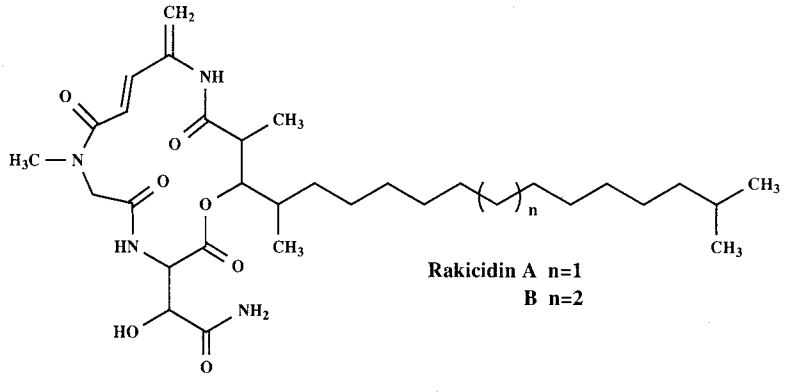
### Microorganism

The producing organism was isolated from a soil sample collected at Andhra Pradesh, India and was identified as a *Micromonospora* sp., strain No. R385-2.

### Media and Culture Conditions

*Micromonospora* strain No. R385-2 was grown on slants of modified BENNETT's medium which contained the following: 0.5% Japanese potato starch (Generichem Corp. Acadama Dextrin No. 3), 0.5% glucose, 0.1% fish meat extract (Mikuni Chem. Industry), 0.1% yeast extract (Difco), 0.2% N-Z Case (Sheffield Products), 0.2% NaCl, 0.1% $CaCO_3$, 1.5% agar. The medium was sterilized at 121°C for 20 minutes. The culture was transferred from the slant into 100 ml of GER medium in a 500 ml flask. The medium contained the following: 2.4% Japanese potato starch, 0.1% dextrose, 0.3% beef extract (BBL), 0.5% tryptone (Difco), 0.5% yeast extract (BBL), 0.2% $CaCO_3$ and was adjusted to pH 7.6 before autoclaving. The medium was sterilized at 121°C for 20 minutes. Frozen vegetative preparations were prepared by mixing a culture grown for 3 days in GER medium with an equal volume of 20% glycerol/10% sucrose, and stored at −80°C. From the frozen stock, 4.5 ml was used as an inoculum into 100 ml of the vegetative medium described above. The culture was grown for 3 days at 28°C at 250 rpm on a rotary shaker, and then 4% (v/v) used to inoculate medium 93A4 which was used as the

Fig. 1. Structures of rakicidins A and B.



production medium. The production medium contained the following: 2.0% Japanese potato starch, 1.0% cane molasses, 1.0% Pharmamedia (Traders Protein), 0.5% corn steep liquor (Sigma), 0.4% $CaCO_3$, 0.04% $MgSO_4 \cdot 7H_2O$, 0.005% $FeSO_4 \cdot 7H_2O$, 0.005% $CuSO_4 \cdot 5H_2O$, 0.0005% $CoCl_2 \cdot 6H_2O$. The culture was grown at 28°C on a rotary shaker at 250 rpm for 8 days.

## Biological Evaluation

### Antitumor Cytotoxicity Assay

A murine cell line M109 (MADISON lung carcinoma 109)[1] was used as a target in the antitumor cytotoxicity assay. The cell line was derived from an *in vivo* grown tumor[2] and was propagated *in vitro* in 175 $cm^2$ Falcon tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ), in culture medium consisting of DULBECCO's modified EAGLE's medium (DMEM, Bio Whittaker, Walkersville, MD) supplemented with 10% Fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS) and antibiotics (Bio Whittaker).

For the assay the cells were removed from the flask with a 0.25% solution of trypsin in HANK's Balanced Salt Solution (Life Technologies, Grand Island, NY) and washed in culture medium. A viable cell count was assessed using a 0.4% solution of trypan blue (Life Technologies). The cells were then plated in 96 well microtiter plates (Corning Glass Works, Corning, NY), $1 \times 10^4$ cells/well in a volume of 180 $\mu$l. The plates were incubated in a $CO_2$ incubator at 37°C for 3 hours to allow cells to adhere. The compounds were then added to the wells at specified concentrations in a volume of 20 $\mu$l, and incubated with the cells for 48 hours. At the end of the incubation period, the viable cells were quantitated using the Neutral Red staining procedure[3]. The cells were incubated for 2 hours with a 40 $\mu$g/ml solution of neutral red (Sigma, St. Louis, MO), fixed for 10 minutes with a 3% solution of formaldehyde (Fisher Scientific, Fair Lawn, NJ), and thoroughly washed with PBS (Bio Whittaker). The dye was eluted from the cells by incubating them for 15 minutes at room temperature with a solution of 50% ethanol (Aaper Alcohol and Chemical Co., Shelbyville, KY) and 1% acetic acid (Fisher Scientific). Dye absorbance was measured at 550 nm using a Thermomax Microplate Reader (Molecular Devices, Sunnyville, CA). The cytotoxic activity was calculated using the following formula:

$$\text{Cytotoxic Activity} = \left(1 - \frac{\text{O.D. sample} - \text{O.D. blank}}{\text{O.D. control} - \text{O.D. blank}}\right) \times 100\%$$

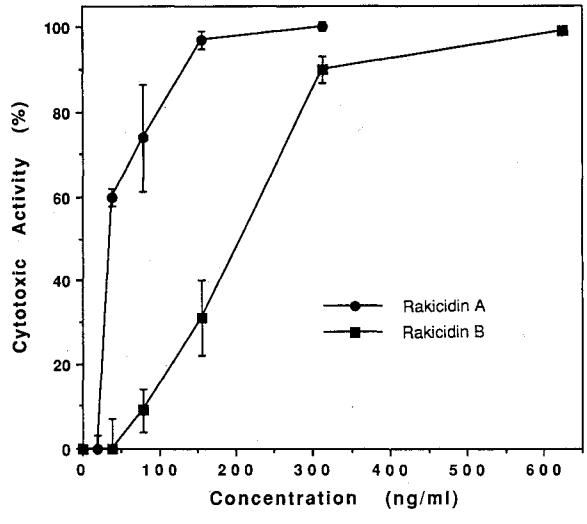
Results presented in Fig. 2 show the cytotoxic activity of rakicidin A and rakicidin B. It can be seen that the activity of rakicidin A was higher than that of rakicidin B. The $IC_{50}$ of rakicidin A was found to be about 40 ng/ml, while that of rakicidin B was 200 ng/ml.

## Isolation and Purification

The isolation of rakicidins A and B is summarized below. The M109 murine MADISON lung carcinoma cell line (*e.g. in vitro* cytotoxicity) was used for bioassay guidance. Fermentation broth (10 liters) prepared as described above was extracted with butanol. The biphasic mixture was vacuum filtered and the organic layer separated and evaporated, yielding 2.5 g.

The crude exract was dissolved in 10% water in methanol and partitioned with hexane. The aqueous methanol phase was diluted to 35% water in methanol and extracted with chloroform. The chloroform extract was chromatographed over diatomaceous earth (dicalite). The bed was eluted in a stepwise fashion with the following solvents: hexane, toluene, ethyl ether, dichloromethane, chloroform, ethyl acetate, acetonitrile, and methanol. The chromatography was monitored by silica gel TLC using anisaldehyde spray reagent with prolonged heating. Rakicidins A and B gave pink-orange spots on a pink background, and were observed as the major components in the dichloromethane, chloroform, ethyl acetate, and acetonitrile fractions. These were pooled and evaporated.

Fig. 2. *In vitro* antitumor activity of rakicidin A and rakicidin B.



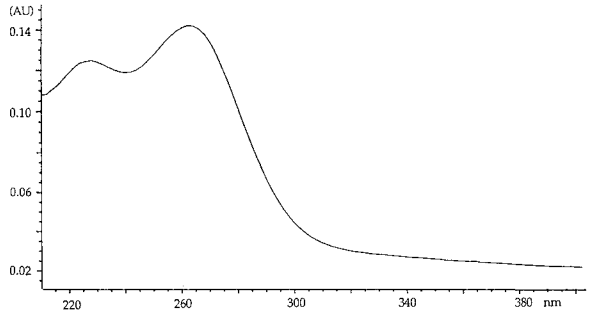

Data from a representative of three independent experiments.

Table 1. Physico-chemical properties of rakicidins A and B.

| | Rakicidin A | Rakicidin B |
|---|---|---|
| Appearance | Colorless amorphous solid | Colorless amorphous solid |
| Molecular formula | $C_{32}H_{54}N_4O_7$ | $C_{33}H_{56}N_4O_7$ |
| FAB-MS ($m/z$) $(M+H)^+$ | | |
| Calcd: | 607.4075 | 621.4211 |
| Found: | 607.4071 | 621.4227 |
| UV (EtOH) | | |
| $\lambda_{max}$ nm ($\varepsilon$) | 262 nm, (9,400)<br>228 nm, (8,200) | 262 nm, (14,200)<br>228 sh nm, (10,200) |
| IR (KBr) $cm^{-1}$ | 1738 (ester C=O), 1696, 1682, 1652, 1618 (amide C=O's) | 1654 (broad, C=O's) |
| $[\alpha]_D$ (Benzene) | −33.5° | Not determined |
| CD $\lambda$ ($\Delta\varepsilon$) (EtOH) | 264 (+1.6), 227 (−19.6) | 263 (+2.4), 227 (−32.0) |
| TLC[a] (Rf) | 0.24 | 0.24 |
| HPLC[b] (Rt) | 26.7 minutes | 27.7 minutes |

[a] Silica gel plates; $CHCl_3$-MeOH (10:2), anisaldehyde spray reagent with heating turns spot pink-orange.
[b] 0.01 M Phosphate buffer and acetonitrile (pH 3.5), MetaO gradient*, C18 column, 1.2 ml/minute, $\lambda$=254 nm.
* According to the method of D. J. HOOK *et al.* (J. Chromatogr. 385, 99 (1987)).

Fig. 3. UV spectrum (EtOH) of rakicidin A.




Further purification was accomplished by silica gel vacuum liquid chromatography (Merck LiChroprep Si 60, 25~40 $\mu$m) using the following step gradient: $CHCl_3$, 1% MeOH in $CHCl_3$, 2%, 5%, 10%, 20%, 25%, 30%, and 50% MeOH in $CHCl_3$. Silica gel TLC revealed the presence of rakicidins A and B in the 20% MeOH in $CHCl_3$ fraction. Final purification was accomplished by C-18 reverse phase preparative HPLC (Rainin Dynamax C18 Column, 21.4 mm × 25 cm L., 8 $\mu$m particle size, 60 Å pore size). The method utilized a linear gradient of 80% aqueous acetonitrile to 100% acetonitrile. Elution flow rate was 10 ml/minute with UV detection at 254 nm. The two major peaks in the chromatogram were collected, yielding rakicidin A (5.1 mg) and rakicidin B (8.7 mg).

## Physico-chemical Properties

Rakicidins A and B were both isolated as colorless amorphous solids. The compounds were soluble in chloroform, methanol, DMSO, and benzene. The molecular formula of rakicidin A was determined to be $C_{32}H_{54}N_4O_7$ by high resolution FAB-MS ($(M+H)^+$ $m/z$ 607.4075; calcd 607.4071), requiring eight degrees of unsaturation. Rakicidin B, differing by one methylene, consists of $C_{33}H_{56}N_4O_7$ (HRFAB-MS $(M+H)^+$ $m/z$ 621.4211; calcd 621.4227). Additional physico-chemical and chromatographic properties for both compounds appear in Table 1.

The UV spectra (Fig. 3) of rakicidins A and B are essentially identical, exhibiting absorption maxima (EtOH) at 228 and 262 nm. In rakicidin A, the IR spectrum (Fig. 4) revealed a hydroxyl absorption at 3420 $cm^{-1}$, an ester carbonyl absorption at 1738 $cm^{-1}$, and amide carbonyl absorptions at 1696, 1682, 1652, and 1618 $cm^{-1}$, suggestive of a depsipeptide. In the $^1H$ NMR spectrum of rakicidin A, (Fig. 5) the resonances were well resolved with minimal signal overlapping, and included 4 NH's, 4 olefinic protons, 1 N-methyl and 4 C-methyl proton signals. Additionally, a large 18-proton resonance ($\delta_H$ 1.23, $\delta_C$ 27.1, 29.2) indicated significant lipophillic character within the molecule. The $^{13}C$ spectrum (Fig. 6) revealed 24 carbon signals, which were attributed to 3 C-methyl groups, 5 aliphatic methylene signals, 6 aliphatic methines, 1 N-methyl, 4 olefinic carbons (1 methylene, 2 methines, and 1 quaternary), and 5 carbonyl groups, thus accounting for seven degrees of unsaturation. Therefore, the remaining degree of unsaturation must be due to a ring structure.

Fig. 4. IR spectrum (KBr) of rakicidin A.

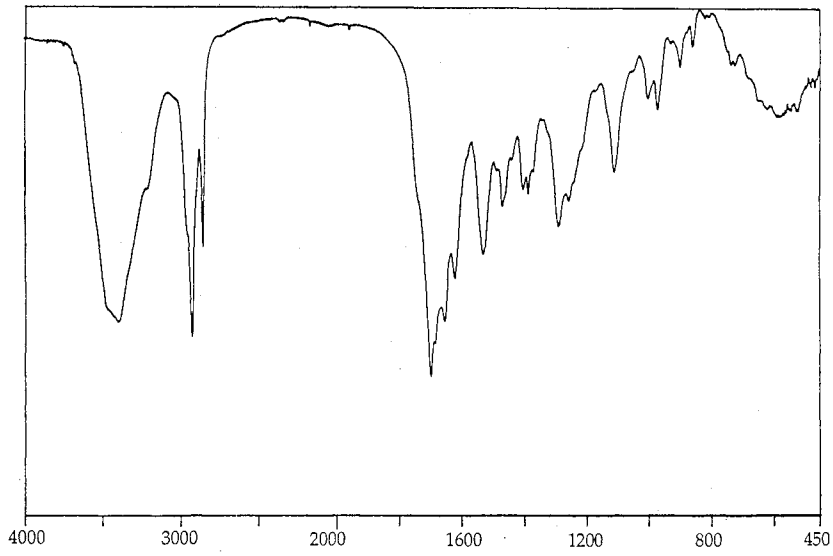



Fig. 5. $^{1}H$ NMR spectrum (500 MHz, DMSO-$d_6$) of rakicidin A.

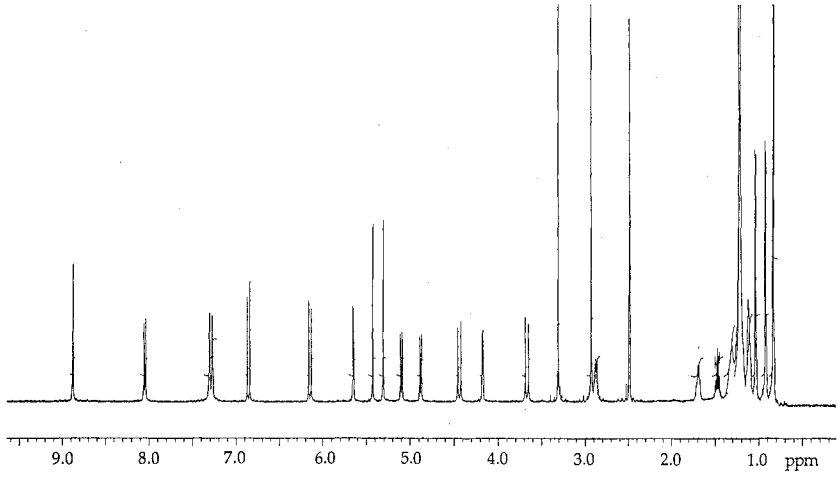
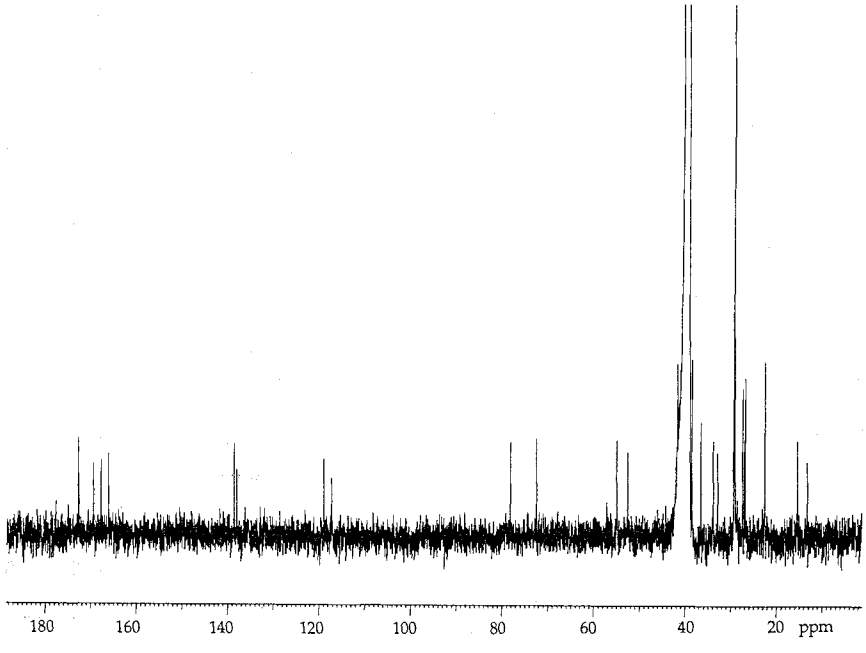



Fig. 6. $^{13}C$ NMR spectrum (500 MHz, DMSO-$d_6$) of rakicidin A.



## Structure Elucidation

A combination of 1D and 2D NMR techniques (COSY, HETCOR, HMBC, and NOEDS) (Table 2) revealed the following structural fragments in rakicidin A: I. 4-amino-penta-2,4-dienoic acid; II. 3-hydroxy-2,4,16-trimethyl heptadecanoic acid; III. Sarcosine; IV. 3-hydroxyasparagine (Fig. 7). In addition, amino acid analysis confirmed the presence of sarcosine and 3-hydroxyasparagine in rakicidins A and B. The following discussion applies to rakicidin A.

I. 4-Amino-penta-2,4-dienoic acid, APDA

The presence of a dienone chromophore was indicated from the UV spectrum ($\lambda_{max}$ (EtOH) 228, 262 nm, $\varepsilon$ 9360, 8220). In the NMR spectrum, a large coupling ($J$= 15.0 Hz) was observed between the two methine protons at $\delta$ 6.15 and 6.86, indicative of a trans-double bond. These protons in turn showed long-range $^{1}H$-$^{13}C$ coupling with an amide carbonyl ($\delta$ 166.2) and the quaternary carbon ($\delta$ 138.1) bearing an exocyclic methylene ($\delta_C$ 117.3, $\delta_H$ 5.31, 5.43).

II. Sarcosine, SAR

The $\alpha$-methylene gem-pair ($J$=18.2 Hz) shows long-range $^{1}H$-$^{13}C$ coupling to the N-methyl carbon ($\delta$ 36.8) and the amide carbonyl ($\delta$ 167.8). Long-range correlations are also observed with the N-methyl protons ($\delta$ 2.94) and the $\alpha$-methylene carbon ($\delta$ 52.8). Evidence linking APDA with SAR was shown by long-range coupling between the $\alpha$-methylene protons of SAR ($\delta$ 3.67 and 4.44) and the amide carbonyl group of APDA ($\delta$ 166.2) and between the amide carbonyl of APDA and the N-methyl ($\delta$ 2.94) of SAR.

III. 3-Hydroxyasparagine, HASP

In the COSY spectrum, the $\beta$-OH group ($\delta$ 5.65) showed coupling with the $\beta$-methine group ($\delta$ 4.18). The

Table 2. Rakicidin A: $^{1}H$ and $^{13}C$ NMR data (DMSO-$d_6$).

| Assignment | $^{13}C$ ppm | $^{1}H$ ppm (mult, $J$ (Hz)) | Long range $^{1}H$-$^{13}C$ correlations | $^{1}H$-$^{1}H$ NOE |
|---|---|---|---|---|
| APDA | | | | |
| C1, CO | 166.2 | — | | |
| C2, CH | 119.0 | 6.15 (d, 15.0) | C1, C4 | H3, C4-NH, SAR-$\alpha$-$CH_2$ HASP-$\alpha$-NH |
| C3, CH | 138.7 | 6.86 (d, 15.0) | C1, C4 | H2, H5 |
| C4, C | 138.1 | — | | |
| C4-N*H* | — | 8.88 (s) | HTHA-C1 | H2, H3, HTHA-H2 |
| C5, $CH_2$ | 117.3 | 5.43 (s) | C3, C4 | H3 |
| | — | 5.31 (s) | C4 | |
| HASP | | | | |
| CO | 172.9 | — | | |
| $\alpha$-CH | 55.0 | 4.88 (dd, 1.8, 10.0) | $\gamma$-CO | $\beta$-CH |
| $\alpha$-NH | — | 8.05 (d, 10.0) | SAR-CO | $\alpha$-CH, APDA-H2, SAR-$\alpha$-$CH_2$ |
| $\beta$-CH | 72.7 | 4.18 (dd, 1.8, 6.2) | | $\alpha$-CH, $\beta$-OH |
| $\beta$-OH | — | 5.65 (d, 6.2) | | $\beta$-CH, $\alpha$-NH |
| $\gamma$-CO | 169.4 | — | | |
| $\gamma$-$NH_2$ | — | 7.31 (s) | $\beta$-C | $\alpha$-NH, $\alpha$-CH, $\beta$-CH, $\beta$-OH HTHA-H6-H14 |
| | — | 7.28 (s) | | $\beta$-CH |
| HTHA | | | | |
| C1, CO | 172.6 | | | |
| C2, CH | 42.0 | 2.89 (d, m, 10.2) | | H19, APDA-C4-NH |
| C3, CH | 78.2 | 5.10 (d, 10.2) | C1, C20, HASP-CO | H4, H19, H20 |
| C4, CH | 34.0 | 1.70 (m) | | H3, H19 |
| C5, $CH_2$ | 33.0 | 1.32 (m) | | |
| | — | 1.12 (m) | | |
| C6-C14, 9×$CH_2$ | 27.1 | 1.23 (m) | | |
| | 29.2 | 1.23 (m) | | |
| C15, $CH_2$ | 38.6 | 1.12 (m) | | |
| C16, CH | 27.5 | 1.48 (m) | | |
| C17, $CH_3$ | 22.6 | 0.83 (d, 6.7) | C15, C16, C18 | |
| C18, $CH_3$ | 22.6 | 0.83 (d, 6.7) | C15, C16, C17 | |
| C19, $CH_3$ | 15.8 | 1.04 (d, 6.9) | C3, C1 | H2, H3, H4 |
| C20, $CH_3$ | 13.5 | 0.92 (d, 6.8) | C3 | H2, H3, H4 |
| SAR | | | | |
| CO | 167.8 | — | | |
| $\alpha$-$CH_2$ | 52.8 | 4.44 (d, 18.2) | N-$CH_3$ | $\alpha$-$CH_2$, APDA-H2 HASP-$\alpha$-NH |
| | — | 3.67 (d, 18.2) | N-$CH_3$, CO, APDA-C1 | $\alpha$-$CH_2$, HASP-$\alpha$-NH |
| N-$CH_3$ | 36.8 | 2.94 (s) | $\alpha$-C, APDA-C1 | $\alpha$-$CH_2$ |

Fig. 7. Partial structures and $^{1}H$, $^{13}C$ chemical shifts (500 MHz, DMSO-$d_6$) of rakicidin A.

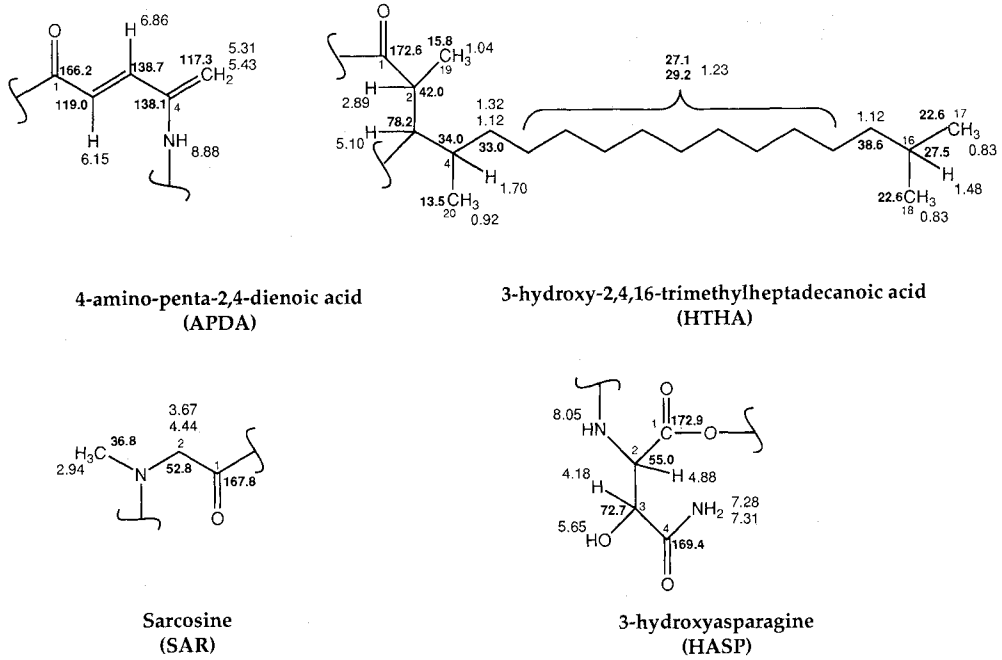
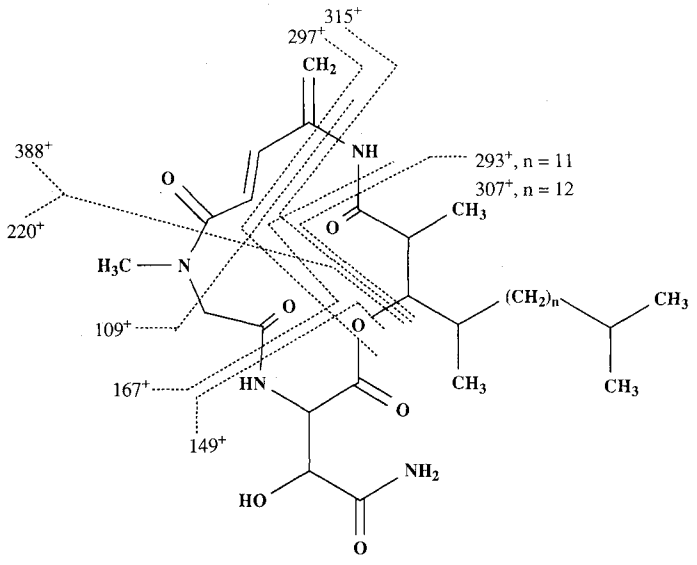



Fig. 8. Summary of mass spectral fragmentations of rakicidins A and B.




α-NH (δ 8.05) showed strong coupling ($J$ = 10.0 Hz) to the α-methine (δ 4.88) which in turn displayed long-range coupling to the γ-carbonyl group at δ 169.4. Long-range coupling was also observed between γ-NH (δ 7.31) and the β-methine carbon at δ 72.7. Long-range coupling between the α-NH at δ 8.05 and the carbonyl at δ 167.8 linked HASP with SAR.

IV. 3-Hydroxy-2,4,16-trimethylheptadecanoic acid, HTHA

In the COSY spectrum, a strong coupling ($J$ = 10.1 Hz) was observed between H-3 (δ 5.10) and H-2 (δ 2.89) which in turn was coupled to the C-19 methyl group (δ 1.04, $J$ = 7.0 Hz). These methyl protons also coupled with the amide carbonyl C-1 (δ 172.6) and C-3 (δ 78.2), while H-3 (δ 5.10) coupled with C-1 (δ 172.6) and the methyl carbon C-20 (δ 13.5). Long-range correlations were observed between the amide proton δ 8.88 of APDA and C-1 (δ 172.6) of HTHA, connecting these two fragments. Furthermore, a strong $^{1}H$-$^{1}H$ NOE was also observed between H-2 (δ 2.89) and C4-NH (δ 8.88) of APDA. An

additional long-range coupling was observed between H-3 ($\delta$ 5.10) of HTHA and the ester carbonyl ($\delta$ 172.9) of HASP, connecting these fragments as well, thus completing the macrocycle. An isopropyl group was indicated by $^1H$-$^1H$ coupling between two degenerate methyl groups (C-17, C-18, $\delta$ 0.83) and a methine (H-16, $\delta$ 1.48). The H-16 methine is coupled to a methylene (H-15, $\delta_H$ 1.12, $\delta_C$ 38.6) which in turn shows coupling with an 18-proton resonance (H-6–H-14, $\delta$ 1.23). The methylene chain is linked to C-5, shown by coupling with gem pair (H-5, $\delta$ 1.12, 1.32). Finally H-5 ($\delta$ 1.12) couples with H-4 ($\delta$ 1.70) which in turn couples with the C-20 methyl protons ($\delta$ 0.92), therefore completing the $\beta$-hydroxy fatty acid spin system.

On the basis of a series of 2D NMR experiments, the gross structure of rakicidin A was determined to be cyclic [APDA-SAR-HASP-HTHA]. Confirmatory evidence for the proposed cyclic sequence of rakicidin A was provided by MS/MS substructure analysis (Fig. 8). The following sequences were observed (clockwise): SAR–APDA (*m/z* 167), APDA–HTHA (*m/z* 388), HASP–SAR (*m/z* 220). The ion (*m/z* 293) was due to the HTHA unit. Rakicidin B displays a nearly identical fragmentation pattern, the only difference being in the presence of ion *m/z* 307 and the absence of ion *m/z* 293. This indicates that the $\beta$-hydroxy fatty acid component in rakicidin B contains one additional methylene group, and consists of 3-hydroxy-2,4,16-trimethyloctadecanoic acid.

## Discussion

The isolation and characterization of two new cytotoxic lipopeptides, rakicidins A and B, are reported. Rakicidins A and B are colorless, amorphous solids, and have a chromophore exhibiting UV absorption at 228 and 262 nm. The macrocycle in rakicidins A and B consists of a tripeptide cyclized through a $\beta$-hydroxy fatty acid. The stereochemistries of the 3-hydroxyasparagine and fatty acid units were not determined. Other reported examples of cytotoxic lipopeptides include the larger, octa-lipopeptides such as halobacillin, from the iturin group[4] and surfactin, a known cytolytic agent[5]. Most recently the stevastelins[6], tetra-lipopeptides cyclized through a $\delta$-hydroxy group of a branched fatty acid, were reported and shown to have immunosuppressant activity. Results presented in the paper show that the cytotoxic activity exhibited by rakicidin A is higher than that exhibited by rakicidin B. It is possible that activity may be a function of the lipid side chain length. However, additional studies would be required to explore this possibility. These compounds showed no antibiotic or antifungal activity, and were also inactive *in vivo* against M109 and P388 murine tumor models.

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