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Characterization of peptidyl-nucleoside antifungal antibiotics from fermentation broth

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SUMMARY

Characterization of sinefungin related antifungal antibiotics from fermentation broth was accomplished by coupling photodiode array (PDA) detection to high performance liquid chromatography (HPLC). From the combined HPLC-PDA evaluation of broth filtrate, we detected five sinefungin related components. Fast atom bombardment (FAB) mass spectroscopic evaluations, mass-analysed ion kinetic energy spectra (MIKES) and collision activated (CA) MIKES of these components confirmed their respective identities. Our findings from the combination of HPLC photodiode array acquisition and FAB-mass spectrometry suggest we have detected the presence of a previously unreported sinefungin analogue.

INTRODUCTION

During our screening for antifungal agents from fermentation broth we identified a *Streptosporangium* sp., Schering culture collection, SCC 1786,[7], that in culture broth produced an antifungal complex. Isolation of the active compounds followed by spectroscopic analysis revealed the presence of peptidyl-nucleosides belonging to the sinefungin family of antibiotics [1,5]. Dehydrosinefungin [6] was identified as the major component. The bioactive complex however consists of at least five components, several of which interconvert during the isolation workup. HPLC coupled with photodiode array detection was developed to detect the presence of these peptidyl-nucleosides in the culture broth. The emergence of photodiode array detector technology that provides uninterrupted UV-VIS spectral acquisition of chromatographic peaks has proven to be an effective tool for discrimination of known reference compounds, structurally similar analogues and metabolites [2,4,8,9,11]. We have used this metho-

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dology to rapidly characterize the presence of sinefungin related nucleosides in two other culture broths: SCC 1927 and SCC 2092.

MATERIALS AND METHODS

Fermentation

The stock culture was maintained as frozen whole broth. A 5% inoculum (3.5 ml) of a thawed suspension was used to inoculate 70 ml of growth medium adjusted to pH 7.5 and consisting of beef extract 0.3%, Tryptone 0.5%, yeast extract 0.5%, cerelose 0.1%, potato starch 2.4%, $CaCO_3$ 0.2%, and tap water 1 liter. After 48 h incubation at 30°C on a rotary shaker operating at 300 rpm, 25 ml (5% inoculum) of the resulting cell suspension was used to inoculate 500 ml of the second stage seed culture in a 2-l Erlenmeyer flask. The culture was incubated as described above. Fermentations were carried out in 500-ml Erlenmeyer flasks containing 350 ml of production medium consisting of PD 650 dextrin 2%, peas 0.5%, Proflo flour 0.5%, liver powder 0.25%, molasses 0.5%, arabinose 1.0%, tap water 1 liter adjusted to pH 7.2 prior to addition of $CaCO_3$ 0.2%, and inoculated with 5% second stage seed culture. The flasks were incubated at 30°C on a rotary shaker operating at 300 rpm for 4–5 days. Production and isolation of the antibiotic were followed by disc diffusion assay against *Candida albicans*.

Isolation

The isolation of the active components is outlined in Scheme 1. The whole broth was treated with Celite and filtered. The anticandidal active complex was adsorbed from the filtrate on Dowex 50 × 4 (H^+ form) and eluted off the resin with 0.5 N NH_4OH. Passage through an Amberlite CG-50 resin column gave two active fractions. The first was non-retained complex I eluting with H_2O. Elution with 0.5 N NH_4OH gave complex II. Complex I and II can be differentiated by their migration behavior on t.l.c. Using silica gel plates and the developing solvent, n-PrOH-conc. NH_4OH (65:35), components of I have R_f 0.05–0.1 and components of II migrate to R_f 0.4–0.5.

Adenine and sinefungin reference standards were obtained from Sigma (St. Louis, MO), and Calbiochem (La Jolla, CA) respectively.

HPLC conditions

An analytical HPLC method to detect the active components required reverse phase ion-pairing conditions as outlined below.

Hewlett-Packard:	HP-1090 liquid chromatographic system.
Column:	Shandon Hypersil ODS (5 μm) 15 cm × 4.6 mm.
Guard Column:	Brownlee C-18 (7 μm) 1.5 cm × 3.2 mm.
Mobile Phase:	0.025 M sodium dodecylsulfate + 0.005 M sodium sulfate (pH 4.7, glacial acetic acid) : acetonitrile (80:20 v/v).
Flow Rate:	1 ml/min.
Detection:	Photodiode array, signals integrated at 220 and 260 nm.
Pressure:	1600 psi.
Temperature:	35°C.

Electrophoretic conditions

Camag HVE System (Camag Scientific Inc., NC, U.S.A.), using Whatman Grade 2 paper; 3000 V, 15 min.

FAB-MS conditions

Full scan FAB mass spectra were obtained on a Finnigan MAT 312 instrument at an ion acceleration voltage of 3 kV. MIKES and CAMIKES were performed using FAB ionization on a VG-ZAB-SE equipment at an acceleration voltage of 8 kV. Helium was used as the collision gas and the pressure adjusted to suppress the main beam intensity, $[M + H]^+$, by about 65%.

The samples were dissolved in water and deposited on a copper probe tip. A thin layer of glycerol/thioglycerol was applied to the tip also and mixed thoroughly with the sample before inserting the probe into the source. Fast atoms of Xe were produced by a saddle field ion source (Ion Tech) operating with a tube current of 2 mA and 6–8 keV energy. All experiments were performed at room temperature.

Scheme 1

Isolation of sinefungin related compounds

Whole broth (10 l)
| filter through celite
Broth filtrate
| sorb on Dowex 50 × 4 (H^+)/elute 0.5 N NH_4OH
Eluate
| amberlite CG-50 (NH_4^+)

H_2O wash — 0.5 N NH_4OH

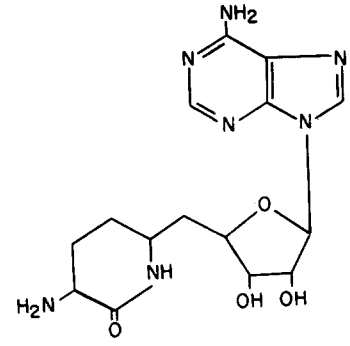
Enriched complex I
1. QAE-sephadex (OA^-c), elute 1 M CH_3COOH
2. SP-sephadex (py^+), elute 0 to 0.2 M py^+
3. Cellulose chromatography elute PrOH-pyridinium acetate[a]
4. MCI CHP2OP gel elute H_2O

Complex I (2 mg)

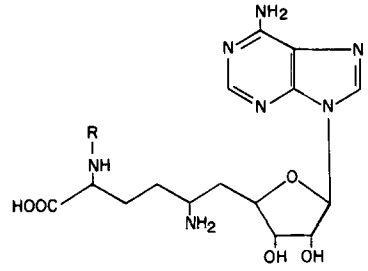
Enriched complex II
CM-sephadex (NH_4^+)
elute 0 to 0.03 N NH_4OH

sinefungin (2 mg) — dehydrosinefungin (5 mg)

[a] 2-Propanol, 0.2 M pyridine, 2 M CH_3COOH-H_2O (3:2:2:1) mixture.

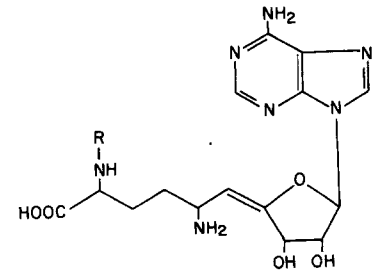


ring-closed sinefungin



sinefungin, R = H

ureido - Sinefungin, R = $CONH_2$



dehydrosinefungin, R = H

ureido-dehydrosinefungin, R = $CONH_2$

RESULTS AND DISCUSSION

An HPLC method was initially developed to detect sinefungin. The mobile phase and pH were then modified in order to detect sinefungin and related compounds from fermentation broth. This was necessary to ensure adequate retention of sinefungin related materials in a region removed from fermentation derived excipients. The selected ion-pair reversed-phase system required careful control of eluent pH. The retention time varied from 15 to over 30 min with a variation of less than 0.5 pH unit. The chromatographic behavior of a sinefungin reference standard, utilizing the above reversed-phase ion-pairing conditions, is shown in Fig. 1a together with an ultraviolet spectrum (inset) obtained on-line during the chromatographic elution. A Dowex 50 × 4 eluate fraction contained a complex of enriched anticandidal components from a *Streptosporangium* culture. Further fractionation using CG-50 (NH_4^+) is shown in Scheme 1. The enriched fraction containing complex II was detected by HPLC during the screening process, as shown in Fig. 1b. The spectral similarity of all components eluting in the 20–30 min window was clearly visible (Fig. 1c). Confirmation of sinefungin was obtained by ultraviolet spectral comparisons (Fig. 1c) of the 21 min peak within the complex using a sinefungin reference standard. Purification by ion-exchange chromatography of several components from the complex and subsequent FAB mass spectral analysis confirmed the presence of sinefungin (21 min) and dehydrosinefungin (22.8 min). Subsequent screening of these nucleosides from other producing cultures SCC 1927 and SCC 2092 required a single cation-exchange pre-concentration step of fermentation broth filtrate using Dowex 50 × 4 (H^+) and elution with 0.5 N NH_4OH prior to HPLC separation and spectral comparison. A typical HPLC separation and detection of components in an 18–25 min window belonging to complex II is shown in Fig. 2.

All the bioactive components are polar, water soluble and amphoteric. They are salts at pH 2–7 (p*K*a) and basic at alkaline pH. The components of complex I convert predominantly into complex II

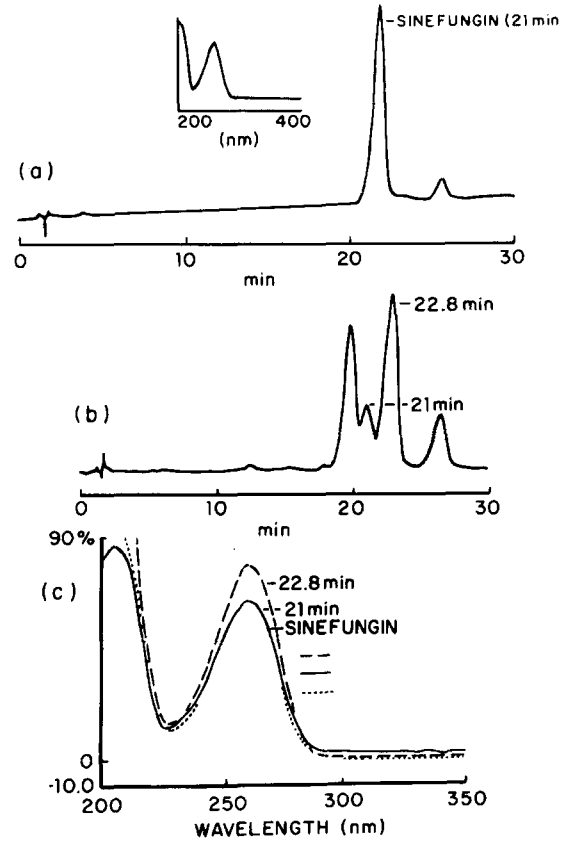


Fig. 1. Chromatographic elution profile (254 nm) of (a) sinefungin (10 μg); with ultraviolet spectrum (inset); (b) chromatographic separation of an enriched complex (10 μg) of anticandidal components from a streptosporangium culture SCC 1786; (c) on-line ultraviolet spectral comparisons of two chromatographic peaks (21, 22.8 min) within the complex with a sinefungin reference (21.8 min).

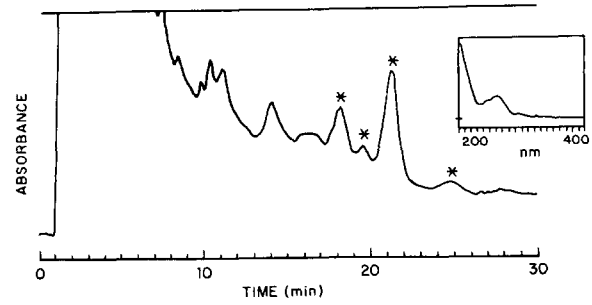


Fig. 2. HPLC-PDA analysis of Dowex 50 (H^+) eluate. The marked peaks indicate the presence of sinefungin related components. The inset indicates a representative UV spectrum for any of the marked peaks.

components during the fermentation process and also under controlled conditions. An aqueous solution of complex I left at pH 6, room temperature, converted to complex II in 3 days. This conversion was easily detected by TLC, and after 3 days, generated the first three peaks in an HPLC sinefungin window (Fig. 1b). During this conversion, the 28 min peak was absent. However, it was also noted that sinefungin converted at room temperature in basic solution to ring closed sinefungin. This conversion was monitored by HPLC; the compound eluted at 28 min. It thus appears that ring closed sinefungin is a minor component belonging to complex II. Ultraviolet and retention time data for the 1st and 3rd (major) peaks were identical to dehydrosinefungin (observed as an isomeric mixture) while the middle peak (minor) was characterized as sinefungin based on UV and retention time characteristics.

Extreme care in the steps used for isolation were taken in order to recover the small amounts (0.5 mg/litre) of bioactive material present in the fermentation. This calculation was based on a 2-fold dilution assay on *C. albicans* in order to determine the quantity of bioactive component present in the broth. Furthermore, the sensitivity of the HPLC method was verified by injecting known concentrations of dehydrosinefungin and establishing the lowest concentration for which the response at 260 nm was greater than 5 MAU. This is the lowest level in which the UV spectrum was clearly representative of an adenine base. This information was then applied to evaluate the Dowex 50 (H^+) eluates with the final stated sensitivity value (0.5 mg/l) obtained by extrapolation to the filtrate level.

Where sufficient material was available from this isolation, the compounds were analysed by spectroscopic methods, UV, NMR, FAB-MS and by hydrolysis experiments. Mass spectrometric (MS) analysis still remains one of the most powerful tools for compound identification. Spectra obtained from soft-ionization techniques such as fast atom bombardment (FAB) are often characterized by intense peaks for the protonated or cationized molecules. This technique was extremely useful in the structural elucidation of these polar compounds.

With regard to complex I, this contains two sinefungin related compounds purified as a mixture according to the steps outlined in Scheme 1, and only characterized by FAB-MS, TLC and paper electrophoresis. Analysis by HPLC was limited since the retention times of the components fall in the 10 min window where many of the other fermentation produced metabolites elute. Acid hydrolysis of complex I material yielded adenine, confirmed by UV comparisons of products from mild hydrolysis of dehydrosinefungin. An identical retention time of 5 min was also observed with an adenine standard by HPLC.

The electrophoretic mobility (pH 2 and 4) of complex I material was found to be exactly one-half the value obtained for sinefungin (see Table 1). This suggested that of the two available amino groups on the ornithine side chain, one must be substituted. FAB mass spectral analysis of complex I obtained after cellulose chromatography revealed that the

Table 1

Electrophoretic mobilities of complex I and II

Electrolyte	pH	Mobility[a]	
		Complex I	Complex II
HCO_2H-CH_3COOH-H_2O (1:3:36)	2	−0.4	−0.8
0.05 M NaH_2PO_4	4.5	−0.2	−0.4
Sodium 0.05 M phosphate	7	0.0	−0.2
Sodium 0.05 M carbonate–bicarbonate	9	+0.2	+0.2

[a] Mobilities relative to vitamin B_{12} (0.0) and *p*-nitrobenzene sulfonate (1.0).

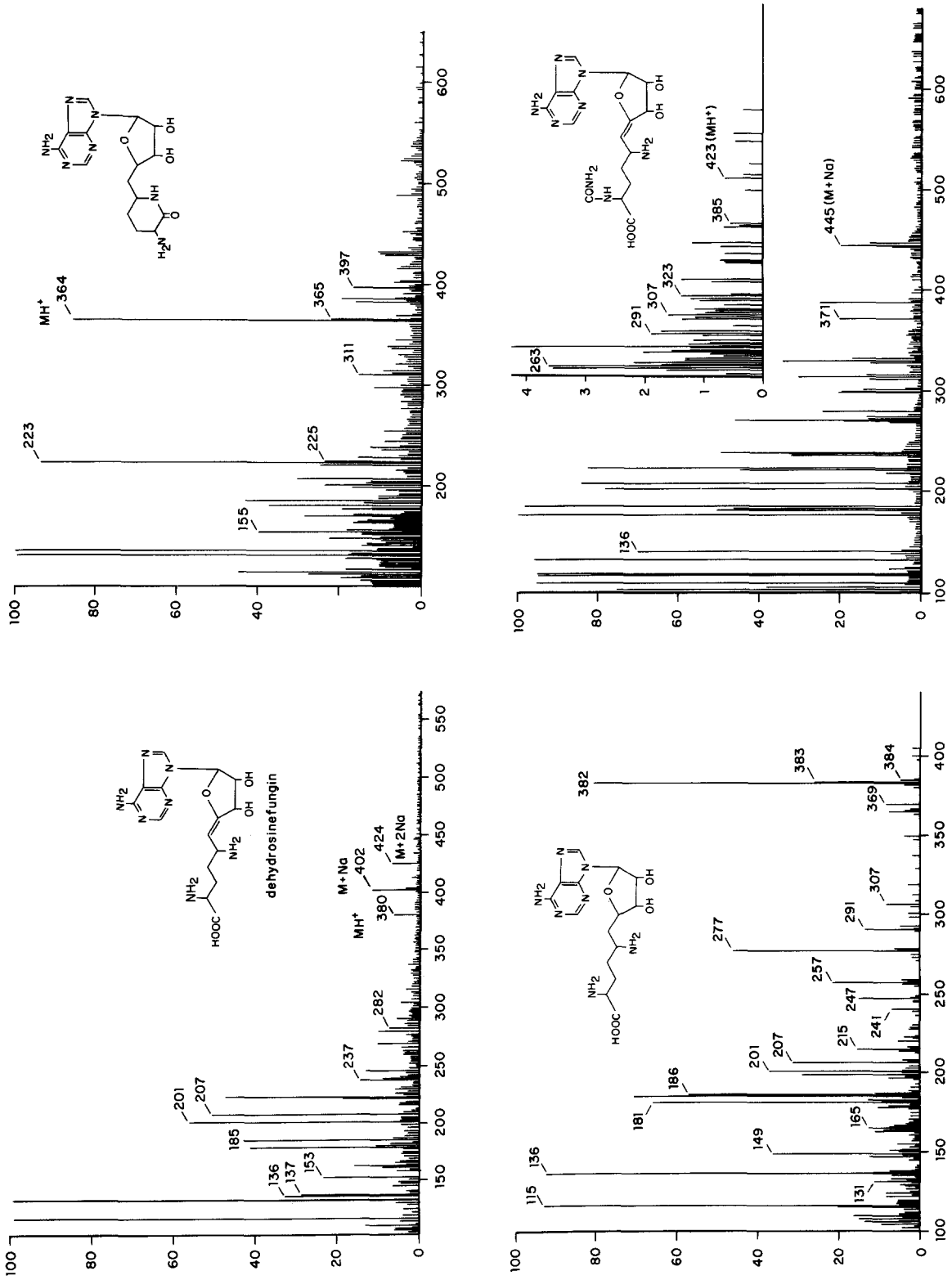


Fig. 3. FAB-mass spectra of sinefungin related compounds.

major component has an ion at *m/z* 445, $[M + Na]^+$, consistent with a sodiated adduct for a compound having a molecular weight of 422, Fig. 3d. Due to the lack of material, no NMR work was performed. From the analytical data, we speculate the structure as the ureido derivative of dehydrosinefungin which to our knowledge has not been previously reported from culture broth. We further speculate that the second minor component in complex I (which converts to sinefungin) is the previously reported ureido sinefungin [6].

The major component in complex II was identified as dehydrosinefungin by FAB-MS which gave $[M + H]^+$ at *m/z* 380, $[M + Na]^+$ at *m/z* 402 and *m/z* 136 (adenine), Fig. 3a. High resolution data confirmed a molecular formula $C_{15}H_{21}N_7O_5$. Mild acid hydrolysis liberated adenine; as expected, no ribose was detected. A minor component present in complex II was sinefungin. The major ion observed was $[M + H]^+$ at *m/z* 382 and $[M + Na]^+$ at *m/z* 404 and *m/z* 136, Fig. 3b. This was confirmed by HPLC comparison with an authentic sample (Calbiochem). It should be noted that Hamill et al. isolated a complex of adenosine containing nucleosides from the culture broth of *Streptomyces griseolus,* and sinefungin was the major product isolated [6]. A trace minor component in complex II was characterized as ring closed sinefungin. FAB-MS analysis indicated $[M + H]^+$ at *m/z* 364 and *m/z* 136, Fig. 3c.

In addition to FAB-MS studies, we carried out mass analysed ion kinetic energy spectral (MIKES) [3] studies in which both selection and identification of the constitutents of a mixture are obtained sequentially. This technique offers the potential for on-line monitoring of routine analyses. On our double focusing reverse geometry VG-ZAB-SE mass spectrometer, fragmentation in the 2nd field free region was monitored by MIKES and CAMIKES using He as the collision gas. Initially this was performed with the pure compound in order to verify daughter ions of the standard. MIKES on $[M + H]^+$, *m/z* 382 produced five major daughter ions and $[MH\text{-}H_2O]^+$, *m/z* 364 was the most intense fragment in the spectrum. The proposed fragmentation pathway of sinefungin and the corresponding MIKES spectrum are shown in Fig. 4. The two fragment ions at *m/z* 136 and *m/z* 247 are particularly important because they reveal the identity of the base, in this case adenine, and the side chain moiety. The spectrum illustrated in Fig. 4 is highly characteristic of the structure of sinefungin.

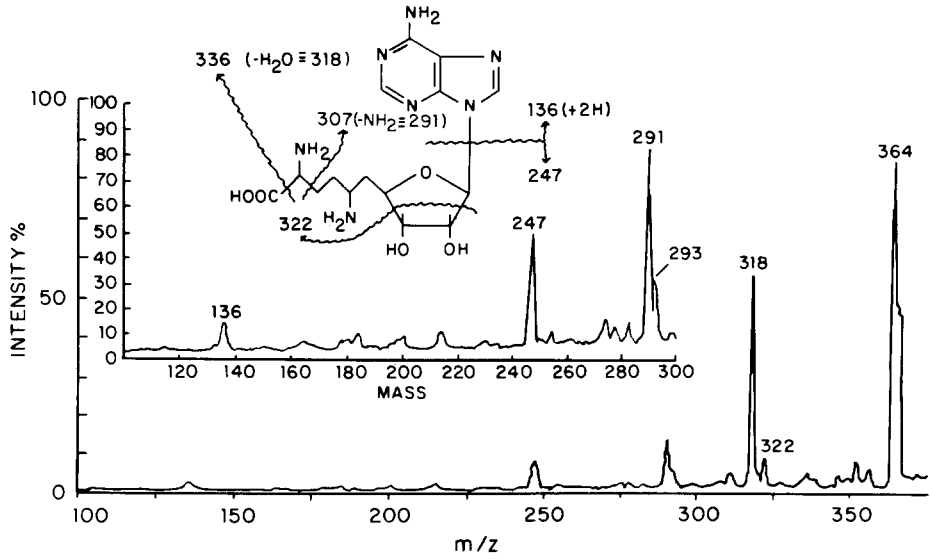


Fig. 4. FAB CA MIKES of sinefungin with helium gas at 65% main beam suppression.

With these experiments it is possible to select a compound from a mixture and identify it on the basis of its characteristic mass spectrum. We attempted to detect the presence of sinefungin in fermentation medium. A level of detection of 400 ppm by the full scan technique was found that is comparable to studies by Tondeur et al. [10] on the detection and identification of toyocamycin, a nucleoside antibiotic from fermentation broth. Although this technique gave useful structural information and confirmed the identity of sinefungin in the matrix, our levels of detection by HPLC were much more sensitive.

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