Purification and Characterization of Roseotoxin B, a Toxic Cyclodepsipeptide from *Trichothecium roseum*

George W. Engstrom,* John V. DeLance, John L. Richard, and Albert L. Baetz

Roseotoxin B, a new mycotoxin isolated from ether extracts of *Trichothecium roseum*, was purified and characterized. It is a cyclodepsipeptide with a molecular weight of 591 and an empirical formula of $C_{30}H_{49}O_7N_5$. It consists of 1 mol each of L-isoleucine, *N*-methylvaline, *N*-methylalanine, *trans*-3-methyl-L-proline, β-alanine, and 2-hydroxy-4-pentenoic acid joined together by amide and ester bonds in a cyclic peptide lactone structure.

Reports concerning the fungus *Trichothecium roseum* Link or its metabolic products have appeared in the literature for many years. Four papers have been published reporting an antagonism between *T. roseum* and some pathogenic plant fungi (Whetzel, 1909; Boning, 1933; Koch, 1934; Greaney and Machacek, 1935). An antifungal compound was isolated from the culture filtrate by Brian and Hemming in 1947. Freeman and Morrison (1948), Freeman and Morrison (1949a,b), and Freeman *et al.* (1949) published papers on isolation, purification, partial characterization, and biological properties of the antifungal metabolite, trichothecin. Structure determination was reported by Fishman *et al.* (1959) and Godtfredsen and Vangedal (1965). Several other metabolites, including diterpene and sesquiterpenoid-type compounds, from *T. roseum* have been isolated, purified, or characterized by Robertson *et al.* (1949), Harris *et al.* (1958), Whalley *et al.* (1959), Scott *et al.* (1964a,b), Holzapfel and Steyn (1968), and Nozoe and Machida (1970).

The first mention of toxicity of extracts of *T. roseum* for animals was reported by Gorlenko (1948) and was based on responses to eye and skin tests in rabbits. In another report on toxicity of trichothecin for animals, Freeman (1955) states that single doses of 250 mg/kg body weight given intravenously to rats caused death. Other reports on the toxicity of *T. roseum* or its metabolites have been published by Bawden and Freeman (1952) and Holzapfel and Steyn (1968).

Richard *et al.* (1969b) have reported results obtained in testing fungal isolates from corn samples collected in central Iowa, including three isolates of *T. roseum*. A fraction was obtained from the diethyl ether extract of rice cultures of the most toxigenic isolate (MC-156) of *T. roseum*, which was toxic to ducklings and mice (Richard *et al.*, 1970). Initial studies to characterize this compound (Richard *et al.*, 1969a) included Kjeldahl nitrogen and an infrared absorption spectrum that indicated that *T. roseum* was a new mycotoxin and not trichothecin or other previously reported metabolites of *T. roseum*.

Results obtained from chromatography of the acid hydrolysate of this mycotoxin support the previous conclusion that this is not trichothecin nor is it a trichothecene type compound (Bamburg and Strong, 1971). This new mycotoxin consists of five ninhydrin-positive molecules and one ninhydrin-negative hydroxy acid arranged in a cyclic lactone structure joined by amide and ester bonds. Compounds of this type are included in a group called cyclodepsipeptides, which frequently have growth-inhibiting properties and have been isolated from bacteria, actinomycetes, and fungi (Russell, 1966; Taylor, 1970).

This paper describes a purification procedure for preparative quantities of the pure crystalline mycotoxin and presents results from continued characterization studies of this new mycotoxin from *T. roseum*. For convenience, we refer to this compound as roseotoxin B, based on the sequence of elution of similar compounds from the silica gel column.

The National Animal Disease Center, Veterinary Sciences Research Division, Agriculture Research Service, U.S. Department of Agriculture, Ames, Iowa 50010.

EXPERIMENTAL SECTION

Materials. The sources of chemicals and supplies were as follows: D- and L-amino acid oxidase, catalase, *N*-methyl-DL-alanine, DL-α-hydroxyisovaleric acid, DL-α-hydroxycaproic acid, the sodium salts of DL-α-hydroxybutyric acid, γ-hydroxybutyric acid, and DL-α-hydroxyvaleric acid from Sigma Chemical Co.; *N*-methyl-DL-valine, L-α-hydroxyisocaproic acid, and the sodium salt of β-hydroxybutyric acid from Calbiochem; fluorescamine from Hoffmann LaRoche, Inc.; *N*-chlorosuccinimide and 3,6-*endo*-methylene-1,2,3,6-tetrahydrophthalic acid from Aldrich Chemical Co., Inc.; *p*-nitrobenzoyl chloride from J. T. Baker Chemical Co.; neutral alumina and silica gel for dry-column chromatography manufactured by M. Woelm of West Germany, distributed by Waters Associates, Inc.; and silica gel H manufactured by E. Merck AG Darmstadt in Germany and distributed by EM Reagents Division of Brinkmann Instruments, Inc. Small quantities of the following proline derivatives have been generously supplied: *cis*- and *trans*-3-methylproline by Dr. A. G. Mauger, Washington Hospital Center, Washington, D.C., and Dr. J. Kollonitsch, Merck, Sharp & Dohme, Rahway, N.J.; a mixture of *cis*- and *trans*-4-methylproline by Dr. G. W. Kenner, University of Liverpool, England; and a mixture of *cis*- and *trans*-5-methylproline from Dr. Edward Katz, Georgetown University, Washington, D.C.

Methods. *Purification Procedure.* Procedures used for growing and extracting *T. roseum* cultures and for toxicological testing of the fractions have been described (Richard *et al.*, 1969b, 1970). Fungal rice cultures from each of 30 flasks were extracted 3 times with 300 ml of diethyl ether. The combined extracts totaled 28 g of a thick oil-like material. The diethyl ether extract (oil) was dissolved in a minimum volume of chloroform and a 20-fold excess of petroleum ether was added, mixed, and allowed to stand at 5° overnight. This solution was filtered to remove insoluble material and concentrated to dryness again. The oil residue was dissolved in diethyl ether to a concentration of about 500 mg/ml. Fifteen milliliters of this solution (7.5 g) was then applied to a 30 × 1.5 cm column of neutral aluminum oxide (activity III), previously equilibrated in diethyl ether. The column was eluted by collecting: (a) ten 30-ml fractions of diethyl ether; (b) ten 30-ml fractions of ethyl acetate; and (c) ten 30-ml fractions of ethyl alcohol.

Roseotoxin B in ethyl acetate fractions 3-6 from three neutral alumina columns were combined and concentrated to dryness, dissolved in diethyl ether, and applied to the 100 × 0.9 cm column of silica gel for dry-column chromatography (activity III) (Loev and Snader, 1965; Loev

and Goodman, 1967). This column was eluted by collecting: (a) ten 30-ml fractions of diethyl ether; (b) ten 15-ml fractions of 80% diethyl ether–20% ethyl acetate; (c) ten 15-ml fractions of 50% diethyl ether–50% ethyl acetate; (d) ten 15-ml fractions of ethyl acetate; and (e) ten 15-ml fractions of ethyl alcohol. The purification procedure is shown as a flow diagram in Figure 1.

Column fractions were monitored on thin-layer chromatography (tlc) plates of silica gel H (0.25 mm) with ethyl acetate as the developing solvent. The various components were visualized by placing the tlc plate into a developing tank containing several crystals of iodine. Chromatographic purity of roseotoxin B was checked on silica gel H plates with three tlc solvent systems: (a) ethyl acetate; (b) chloroform–methanol (97:3, v/v); and (c) 3-pentanone–acetone (50:50, v/v). All solvents were analytical reagent or reagent grade except 3-pentanone. Chloroform and ethyl acetate were redistilled before use. Roseotoxin B was recrystallized from ether–petroleum ether. A sample of crystalline roseotoxin B was submitted to an independent laboratory (C. J. Mirocha, University of Minnesota) for gas chromatographic–mass spectroscopic analysis. The melting point was determined with the sample in a sealed capillary tube and heated to give a temperature increase of 1°/min.

Elemental and Spectral Analyses. Elemental analyses were determined by the Galbraith Laboratories of Knoxville, Tenn. Ultraviolet and visible range absorption spectra were obtained with a Zeiss PMQ II spectrophotometer. Infrared spectra were obtained with a Beckman IR-9 spectrophotometer and the samples were prepared as KBr pellets. Nuclear magnetic resonance (nmr) spectra were determined with a Varian 100-MHz Model HA-100 spectrometer and carbon-13 nmr spectra with either a Varian Model CFT-20 or an XL-100 spectrometer. The molecular weight, elemental composition, and fragmentation patterns of roseotoxin B and of two unknown hydrolysis products were determined by a Model MS-30 double focusing, high-resolution, mass spectrometer of Associated Industries at Shrader Analytical and Consulting Laboratories, Detroit, Mich.

Acid Hydrolysis of Roseotoxin B. One sample of roseotoxin B (4 mg) was hydrolyzed by refluxing with 6 *N* HCl for 24 hr, another (10 mg) by autoclaving with 6 *N* HCl in a sealed glass vial for 72 hr, and a third by refluxing with 8 *N* H_2SO_4 for 24 hr (28 mg). Excessive HCl was removed by flash evaporation. Sulfate ions were precipitated by titration with a barium chloride solution to pH 5. The samples were analyzed with a Model 121 Beckman automatic amino acid analyzer following the procedure described by Spackman *et al.* (1958) for physiological fluids with a 55-cm column of Beckman type PA-28 custom spherical resin. The buffer flow rate was programmed at 50 ml/hr and ninhydrin at 25 ml/hr unless otherwise indicated. A slight modification of the temperature program changed the temperature to 56° subsequent to the buffer change.

Barium Hydroxide Treatment for a Lactone. A 4-mg sample of roseotoxin B was treated with 0.32 *N* $Ba(OH)_2$ at 25° for 20 hr and the barium ions were precipitated by titration with 2 *N* H_2SO_4 to pH 5. Insoluble barium sulfate was removed by centrifugation (Sheehan *et al.*, 1968). The KBr pellet was prepared with 1 mg of dry sample plus 300 mg of KBr.

Hydroxylamine–Ferric Chloride Test for Lactone. This test for lactones, esters, and amides of carboxylic acids was done following the procedure described by Whittaker and Wijesundera (1952).

Thin-Layer Chromatography of Acid Hydrolysate. Individual components of the acid hydrolysate were isolated and purified by tlc. The solvent systems used for the ninhydrin positive components were (a) 1-butanol–acetic acid–water (60:20:20, v/v/v); (b) 1-propanol–ammonium hydroxide (80:20, v/v); (c) ethanol–water (90:10, v/v); and for the hydroxy acid they were (a) petroleum ether–diethyl ether–acetic acid (60:40:2, v/v/v) and chloroform–methanol–acetic acid (80:20:2, v/v/v). Thin-layer plates were prepared with silica gel H at a thickness of 0.5 mm, and an aqueous solution of the acid hydrolysate was applied to the plates in the form of a streak 3 cm above the bottom of the plate. After development of the tlc plates in the solvent system and air drying, narrow vertical bands scribed on each side of the tlc plates were sprayed with ninhydrin or 2′,7′-dichlorofluorescein in order to visualize the respective bands and facilitate scribing the horizontal lines before scraping each band from the plates. The samples were eluted from the silica gel powder with water and filtered, and the volume was reduced by flash evaporation.

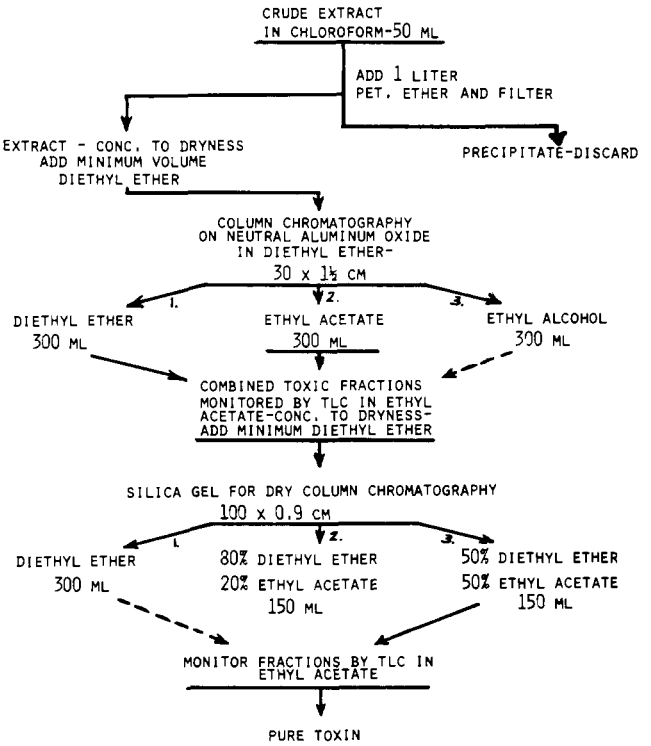

Figure 1. A flow diagram of the purification procedure for roseotoxin B, a new mycotoxin, from *Trichothecium roseum*.

Chromic Acid Oxidation. Chromic acid oxidation of roseotoxin B and of alkali-treated roseotoxin B was done following the procedure described by Sheehan *et al.* (1958).

Tests for N-Alkyl Amino Acids. The samples to be tested were spotted on filter paper and dipped first in a 0.2% solution of *p*-nitrobenzoyl chloride in benzene and then in a 10% solution of pyridine in petroleum ether following the procedure described by Plattner and Nager (1948).

A second test involved applying the sample to filter paper and adding a drop of a nitroprusside–acetaldehyde reagent. The appearance of a blue to violet color indicated a positive test (Feigl and Anger, 1937).

Tests for Cyclic Imino Acids. Aqueous solutions of the samples to be tested were combined with 0.15% ninhydrin in glacial acetic acid and placed in a boiling water bath for 35 min or left at room temperature for several hours following the procedure described by Piez *et al.* (1956).

Amino Acid Oxidase Experiments. The enzyme L-amino acid oxidase from snake venom (*Crotalus adamanteus*) was used to assay for L-amino acids in the acid hydrolysate according to a modification of the procedure of Wellner and Meister (1960). D-Amino acid oxidase from pig kidney was used according to a modification of the procedure of Burton (1955). The volume of the incubation solutions was reduced by flash evaporation before thin-layer chromatography. The silica gel plate was developed in benzene–eth-

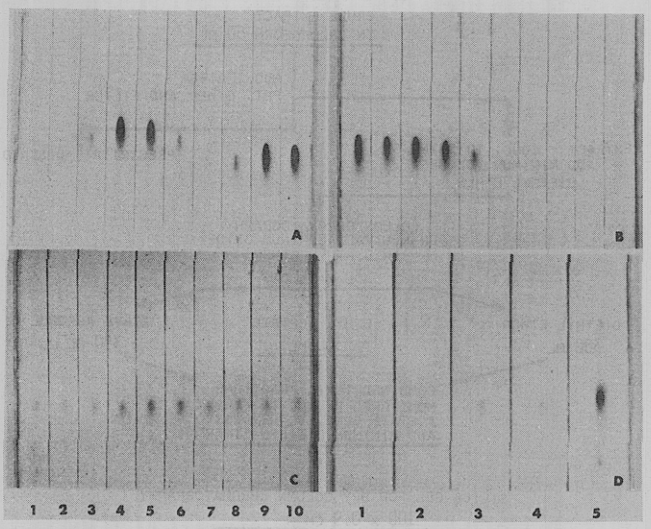

Figure 2. Thin-layer chromatography of all but the first ten fractions from elution of the silica gel column showing resolution between rosetoxin A (fractions 3–6, panel A), roseotoxin B (fractions 8–10, panel A, and 1–5, panel B), and roseotoxin C (fractions 1–10, panel C).

anol–ammonium hydroxide (28:56:16, v/v/v), air dried, sprayed with ninhydrin, and heated at low temperature to develop the color.

Gas Chromatography. A Beckman gas chromatograph (Model GC-4) equipped with a flame-ionization detector was used. The 12 ft × ⅛ in. stainless steel column was packed with 80–100 mesh, acid-washed Chromosorb W coated with 10% SP1200/1% H_3PO_4 (Supelco, Inc., Bellefonte, Pa.). Helium (zero grade) was used as the carrier gas at a flow rate of 36 ml/min. Water pump compressed air (300 ml/min) and ultrahigh purity hydrogen (38 ml/min) were supplied through dual pressure controllers that maintain constant pressure of the gases to the burners of the flame ionization detector. Columns were conditioned overnight at 200° and used isothermally at 180° for gas chromatography of the free hydroxy acids. The inlet temperature was 220° and the detector temperature was 250°. Aqueous samples (microliters) of standard hydroxy acids (25 mg/ml) and the unknown hydroxy acid were injected directly on column by glass syringe. Chromatograms were recorded on the Beckman 10-in., 1-mV potentiometric recorder.

Methyl esters of hydroxy acids were prepared by refluxing a mixture of boron trifluoride in methanol with the standard hydroxy acid or dried acid hydrolysate from 200 mg of roseotoxin B for 1 hr. Water was added and the mixture was extracted with diethyl ether or chloroform to isolate the methyl ester. The esters were dissolved in methanol and chromatographed at 120° with an inlet temperature of 170° and a detector temperature of 220°.

RESULTS AND DISCUSSION

In a review on microbial peptide lactones, Taylor (1970) states that it is sometimes very difficult to isolate a single metabolite of this type free of its closely related metabolites and to define the criteria of purity. A preliminary procedure for isolation of roseotoxin B was not satisfactory. Modification of the procedure to include column chromatography with a special silica gel (100 × 0.9 cm) in place of preparative tlc provided much better resolution of roseotoxin B from other closely related metabolites. The purified toxin is crystalline and has a sharper and higher melting point (199–200°) than that obtained for the amorphous solid isolated by the preliminary procedure (172–175°). In a review on cyclodepsipeptides, Russell (1966) states that the pure compounds are crystalline and optically active and have moderately high melting points.

Purification. The first column was made up with neutral aluminum oxide (activity III) and was eluted with 300 ml of diethyl ether. The diethyl ether fractions contained most of the compounds present in the crude extract but did not contain any roseotoxin B. The second elution solvent, ethyl acetate, eluted roseotoxin B and related compounds for a total of 1500 mg or 6.7% of the original crude extract. Elution of the silica gel column according to the prescribed schedule provided an excellent separation of preparative quantities of roseotoxin B from other compounds eluted before and after it (Figure 2). Column fractions 3–6 in panel A of Figure 2 contained roseotoxin A, fractions 8–10 in panel A and 1–5 in panel B contained roseotoxin B. Column fractions 1–10 in panel C and 1–5 in panel D contained still other closely related metabolites, including roseotoxin C, which were eluted with ethyl acetate and ethyl alcohol. A total of 300–350 mg of roseotoxin B was obtained from processing 22.5 g of crude extract with this new procedure. Fractions containing chromatographically pure roseotoxin B were combined and the solvent removed by flash evaporation. This gave a thick clear syrup that slowly turned to a white crystalline solid after standing for a few days. Recrystallization of this material from ether–petroleum ether resulted in formation of white needle-like crystals that had a melting point of 199–200°. Results obtained from gas chromatographic–mass spectroscopic analysis of roseotoxin B demonstrated that there was no contamination with any known trichothecene type compound. In addition, the symptoms of animals which received roseotoxin B (Richard *et al.*, 1970) are distinctly different from those produced by trichothecene type toxins.

Molecular Weight and Elemental Analysis. Molecular weight and exact mass values determined by high-resolution mass spectroscopy were *m/e* 591 and 591.3685, respectively. An empirical formula of $C_{30}H_{49}O_7N_5$ was determined by calculations using the exact mass of roseotoxin B and of the constituent elements. The per cent composition of each element in the empirical formula was 60.87% carbon, 8.35% hydrogen, 18.90% oxygen, and 11.83% nitrogen. These values are in very good agreement with those found by analysis of the crystalline compound, 60.79% carbon, 8.25% hydrogen, 19.43% oxygen, 11.72% nitrogen, and 0% sulfur, thereby confirming the accuracy of the empirical formula.

Infrared Spectrum. Major peaks in the infrared spectrum of roseotoxin B are consistent with those obtained for cyclodepsipeptides (Russell, 1966; Taylor, 1970). These include: the ester lactone absorption at 1730 cm^{-1}; the amide carbonyl peaks at 1680–1635 cm^{-1}; the amide II band at 1525 cm^{-1}; the N–H stretching peak at 3380 cm^{-1}; and the C–O stretch for lactone at 1180 cm^{-1} (Figure 3). The disappearance of these peaks is strong evidence in support of a lactone structure (Nakanishi, 1962). In addition, the hydroxylamine–ferric chloride test for lactones of carboxylic acids gave a positive test (brown color).

Chromic Acid Oxidation. Chromic acid oxidizes compounds that have free primary or secondary but not tertiary hydroxyl groups. Failure to observe chromic acid oxidation of roseotoxin B until after lactone hydrolysis indicates that there are no free hydroxyl groups in roseotoxin B. Oxidation of roseotoxin B after lactone hydrolysis demonstrates that the hydroxyl function must be primary or secondary. Samples were hydrolyzed by refluxing with 6 *N* HCl for 24 hr and chromatographed on tlc plates with benzene–ethanol–ammonium hydroxide (28:56:16, v/v/v). Visualization of the tlc plate in an iodine jar demonstrated that the alkali-treated sample of roseotoxin B with a free hydroxyl group contained one less spot than the control sample which was not treated with alkali. The hydroxy compound moved ahead of isoleucine and was well resolved from all ninhydrin positive components.

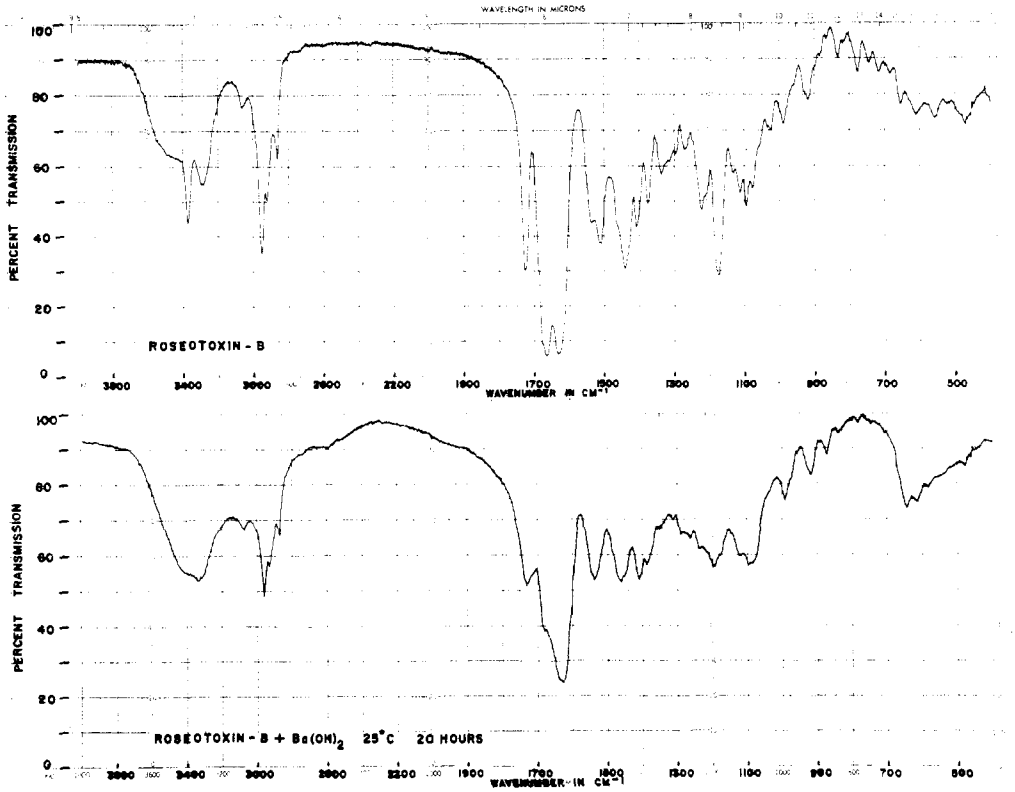

Figure 3. Infrared absorption spectra of crystalline roseotoxin B (upper) and $Ba(OH)_2$ treated roseotoxin B (lower). Samples were prepared as KBr pellets.

Solubility and Specific Rotation. The solubility characteristics of roseotoxin B are consistent with those expected for a cyclic structure of this type. It is soluble in 95% petroleum ether–5% chloroform, carbon tetrachloride, chloroform, ether, ethyl acetate, and ethyl alcohol, has limited solubility in warm water at 40°, and is insoluble in petroleum ether.

The specific rotation of roseotoxin B in ethyl alcohol was $[\alpha]^{25}D$ $-235 \pm 2°$.

Amino Acid Oxidase Experiments. L- and D-amino acid oxidases were assayed to determine the stereochemistry of the amino acids from the acid hydrolysate. Snake venom L-amino acid oxidase oxidized isoleucine, but porcine kidney D-amino acid oxidase did not oxidize any of the ninhydrin-positive compounds from the acid hydrolysate. Coggins and Benoiton (1970) have reported that amino acid oxidase assays are not applicable to *N*-methyl amino acids because suitable enzymes are not available.

Ultraviolet Spectrum. A solution of roseotoxin B in ethyl alcohol was transparent to ultraviolet light with a wavelength higher than 220 nm. Ultraviolet absorption between 215 and 200 nm is due in part to the fact that roseotoxin B is a peptide and peptides exhibit selective absorption in that region of the spectrum (Bailey, 1967 and Waddell, 1956).

Ion-Exchange Chromatography of Acid Hydrolysates. Samples of acid hydrolysates and standard amino acid mixtures were chromatographed on the amino acid analyzer which was programmed for fractionation of physiological fluids (Spackman *et al.*, 1958). Some changes were made in first buffer pH, buffer flow rate, and sample concentration in an effort to help characterize unknown peaks. Ion-exchange chromatograms in Figure 4 were obtained with a first buffer pH of 3.29 and buffer flow rate of 50 ml/hr. The last two peaks in the upper chromatogram have the same elution times as isoleucine and β-alanine in the standard mixture.

When a more concentrated sample was chromatographed, with a first buffer pH of 3.23, the shape of the peak with an elution time of 97 min was distorted. This peak had the same elution time and distorted shape as standard *N*-methyl-DL-alanine (sublimed) under the same conditions. Ion-exchange chromatography of the same acid hydrolysate plus standard *N*-methyl-DL-alanine resulted in an increase in size of the distorted peak, and no additional peaks compared with those in the control acid hydrolysate chromatogram.

Ebata *et al.* (1966) have reported that the peaks for *N*-methyl amino acids, in general, have good symmetry except for *N*-methyl derivatives of isoleucine, alloisoleucine, leucine, valine, and cystine. In 1970, Coggins and Benoiton published a report showing the distorted shape of *N*-methylglycine (sarcosine), *N*-methylalanine, and *N*-methylleucine peaks obtained under normal operating conditions of an automatic amino acid analyzer. Coggins and Benoiton (1970) also reported that reducing buffer flow rates by one-half resulted in large increases in peak size of *N*-methyl amino acids. They demonstrated that the increase in the size of the peak was due to the longer time the ninhydrin–*N*-methyl amino acid mixture spent in the reaction coil in the boiling water bath and that this additional time was necessary to obtain maximum color yields from this reaction. When the acid hydrolysate was chromatographed at one-half buffer flow rate (lower chromatogram, Figure 5), the first peak was increased in size to the extent that it was now larger than the second peak. This contrasts with the chromatogram at the standard buffer flow rate (upper chromatogram, Figure 5), where the first peak was much smaller than the second peak.

Ebata *et al.* (1966) reported that the *N*-methyl derivative of valine was eluted from a 50-cm ion-exchange column just slightly after *N*-methylalanine. An authentic standard sample of *N*-methyl-D,L-valine was added to the acid hydrolysate of roseotoxin B and chromatographed following the procedure of Spackman *et al.* (1958). Only one peak increased in size and its' elution time was the same as that for the double peak in Figure 6. We conclude that both *N*-methylalanine and *N*-methylvaline are present in

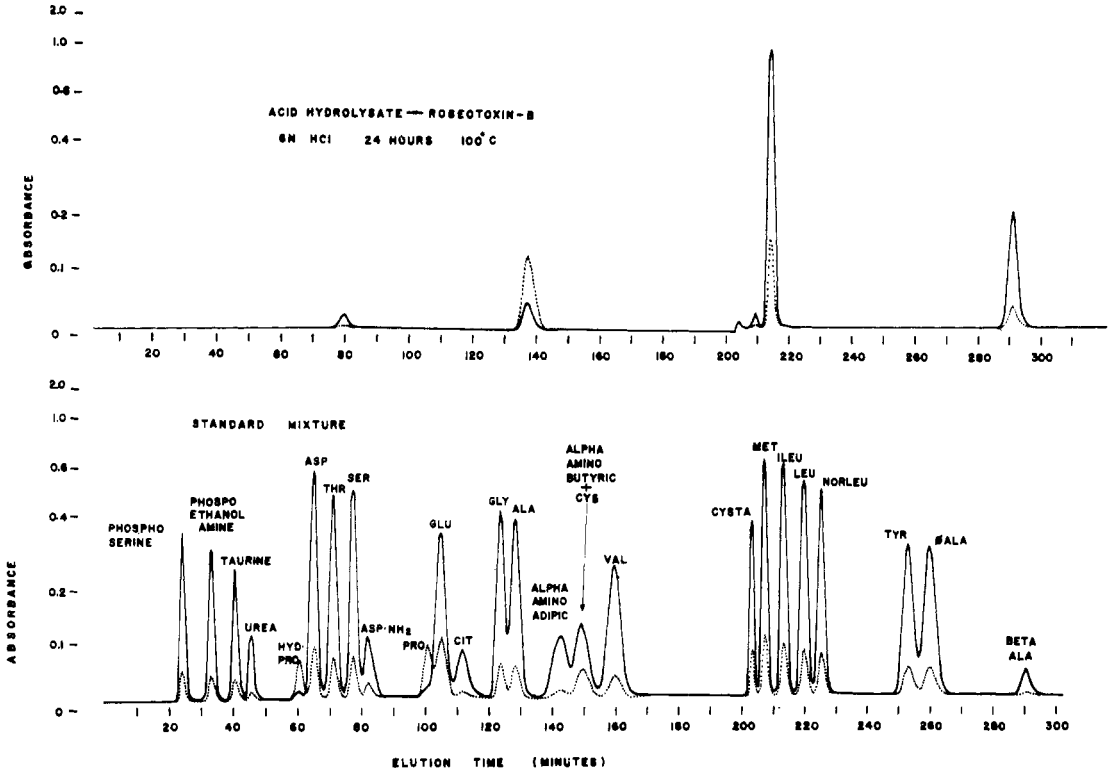

Figure 4. Ion-exchange chromatograms of a 24-hr 6 *N* HCl hydrolysate of roseotoxin B (upper) and of a standard mixture of amino acids (lower) on a 55-cm column at 30 and 56° with a buffer flow rate of 50 ml/hr and first buffer pH of 3.29.

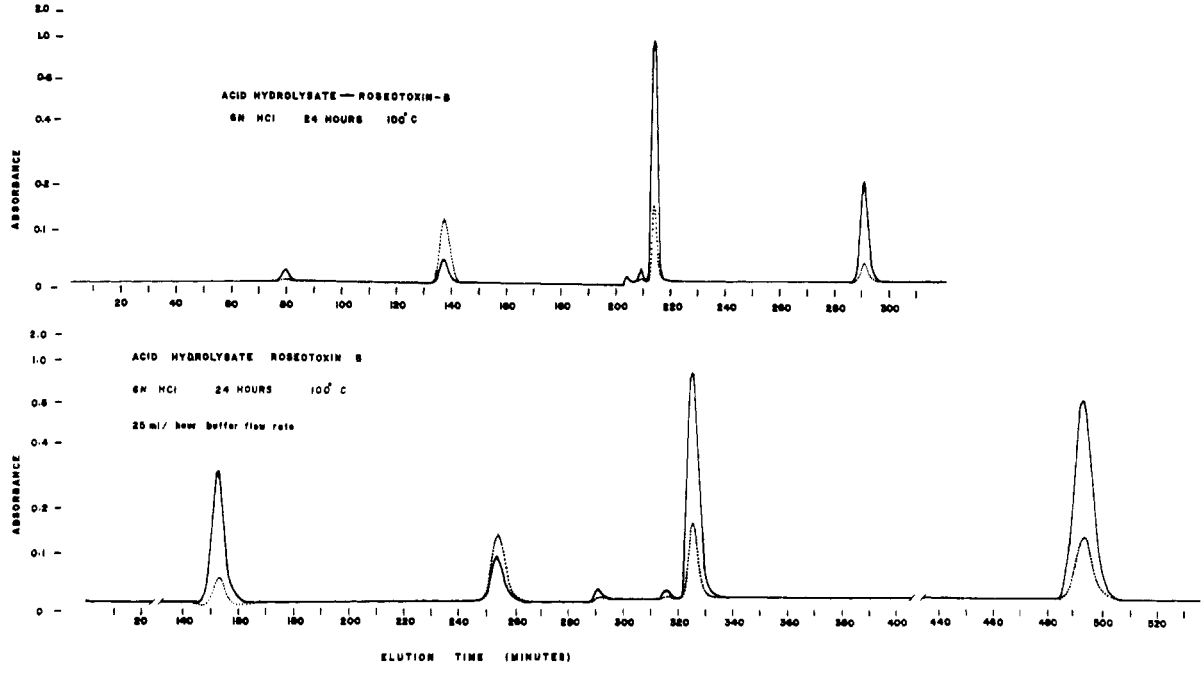

Figure 5. Ion-exchange chromatograms of a 24-hr 6 *N* HCl hydrolysate of roseotoxin B on a 55-cm column at 30 and 56° with a first buffer pH of 3.29 and buffer flow rate of 50 ml/hr (upper) and 25 ml/hr (lower).

the acid hydrolysate and appear in the ion-exchange chromatogram as the double peak (Figure 6) (elution time = 85–98 min).

The elution time for the ninhydrin yellow peak with pH 3.23, 0.2 *M* citrate buffer was 155–158 min and with pH 3.29 buffer it was 137–138 min. The 440/570 nm ratio for this peak was 2.5–2.7 with pH 3.23 buffer and 2.8–3.1 with pH 3.29 buffer.

A review of proline derivatives that might correlate with these data focused on methylproline. A quantitative method for cyclic imino acids (Piez *et al.*, 1956) also provided some qualitative information about the visible range spectrum of the stable colored complex that they form with ninhydrin in glacial acetic acid. When the component that forms a yellow product with ninhydrin was used, the spectrum contained one absorption maxima at 350 nm. Waldman and Nishimura (1968) reported that the ninhydrin-3-methylproline color complex formed in glacial acetic acid has an absorption peak at 350 nm. In 1966, Mauger *et al.* reported elution volumes of 187 ml for *cis*-3-methylproline, 230 ml for alanine, and 233 ml for *trans*-3-methylproline from a 150-cm column operated at

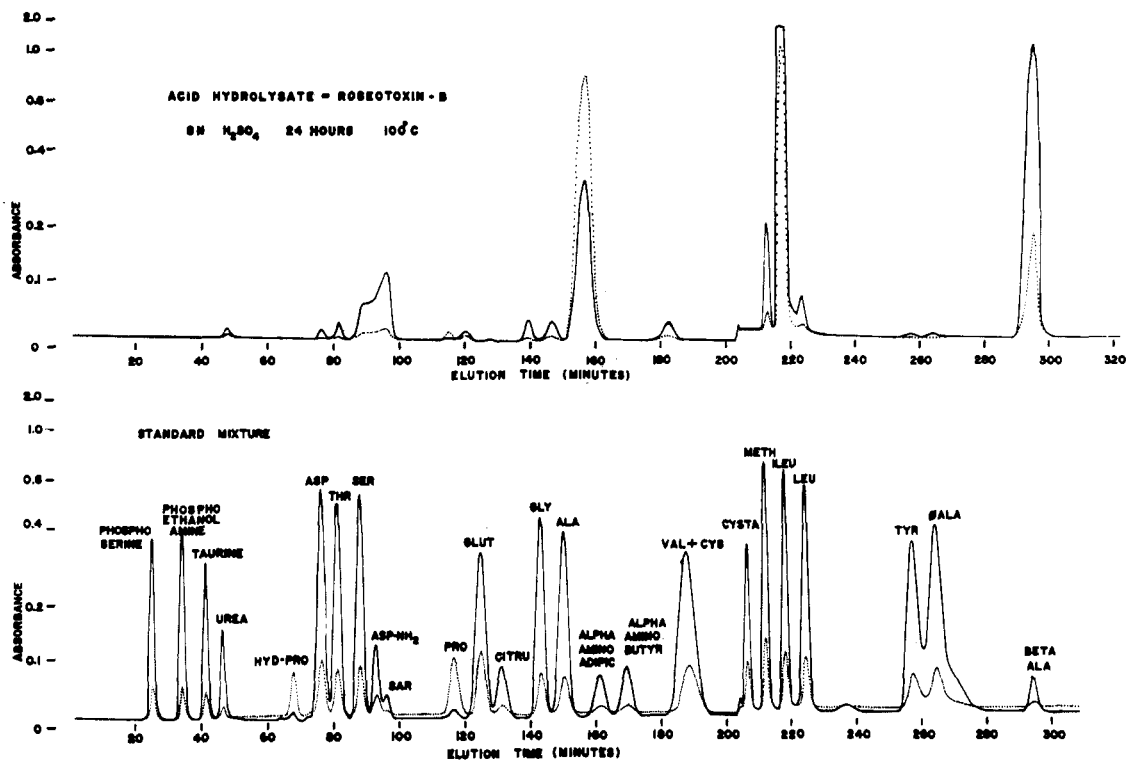

Figure 6. Ion-exchange chromatograms of a 24-hr 8 *N* H_2SO_4 hydrolysate of roseotoxin B (upper) and of a standard mixture of amino acids (lower) on a 55-cm column at 30 and 56° with a buffer flow rate of 50 ml/hr and first buffer pH of 3.23.

50° beginning with pH 3.25, 0.2 *M* citrate buffer. The elution volume for standard *trans*-3-methylproline in our ion-exchange system was 137 min which was just beyond that for alanine (128 min) in the standard mixture. This was interpreted as suggestive evidence that the ninhydrin yellow peak might be *trans*-3-methyl-L-proline. Recently, Mauger *et al.* (1973) published another report, including an ion-exchange chromatogram showing separation of the isomeric methylprolines with pH 3.05, 0.2 *M* citrate buffer at a buffer flow rate of 34 ml/hr. Under these conditions, elution times were 229 min for *cis*-3-methylproline and 280 min for *trans*-3-methylproline. It is difficult to compare elution times and volumes with literature values because there is usually at least one important variable that does not match the conditions being used.

Standard samples of *cis*- and *trans*-3-methylproline, a mixture of *cis*- and *trans*-4-methylproline, and a mixture of *cis*- and *trans*-5-methylproline were chromatographed separately on the amino acid analyzer with pH 3.29, 0.2 *M* citrate buffer. The elution time of standard *trans*-3-methyl-L-proline was 137 min, which was the same as that for the peak that forms a yellow product with ninhydrin (Figure 5). The elution times were 93 min for *cis*-5-methylproline, 102 min for *trans*-5-methylproline, 105 min for *cis*-3-methylproline, and 124 min for racemic 4-methylproline. A sample of standard *trans*-3-methyl-L-proline was then added to the acid hydrolysate and chromatographed; the conditions were the same as those described above. The size of the ninhydrin yellow peak (elution time of 138 min) was increased without loss of symmetry, and there was no new peak anywhere on the chromatogram. The 440/570 nm ratio for the ninhydrin yellow peak varied from 2.5 (pH 3.23 buffer) to 3.0 (pH 3.29 buffer) and was the same as that ratio for standard *cis*- and *trans*-3-methylproline. The 440/570 nm ratio was 17.1 for 4-methylproline and 6.2–6.5 for 5-methylproline.

When the concentration of the acid hydrolysate was increased, a few other peaks appeared. The smaller ninhydrin yellow peak with an elution time of 118 min with pH 3.23 buffer and 105 min with pH 3.29 buffer was shown to be *cis*-3-methyl-D-proline on the basis of these elution times compared with those of the standard under the same conditions. *cis*-3-Methyl-D-proline can form from *trans*-3-methyl-L-proline by epimerization under certain conditions similar to those of acid hydrolysis (Kollonitsch *et al.*, 1966). The peak that appears immediately before isoleucine was probably alloisoleucine rather than methionine because no sulfur was detected on elemental analysis. In addition, Piez (1954) has reported that alloisoleucine is eluted just before isoleucine, and Moore *et al.* (1958) report that after 72 hr of acid hydrolysis, as much as 5% of the isoleucine may be changed to the allo form as a result of racemization at the β carbon.

Thin-Layer Chromatography of the Acid Hydrolysate. One ninhydrin-negative and five ninhydrin-positive components of the acid hydrolysate of roseotoxin B were purified by tlc in sufficient quantity for chemical characterization studies.

Isoleucine. The fastest moving ninhydrin-positive component of the acid hydrolysate in all the thin-layer solvent systems used here was identical with an authentic standard sample of isoleucine and had an infrared spectrum that was identical with the spectrum of standard isoleucine.

β-Alanine. The second fastest moving ninhydrin-positive component in the butanol–acetic acid–water (60:20:20, v/v/v) solvent system was blue when treated with ninhydrin and was identical with an authentic standard sample of β-alanine. An infrared spectrum obtained on the purified sample was identical with one of standard β-alanine.

N-Methylalanine. The slowest moving ninhydrin-positive component in butanol–acetic acid–water (60:20:20, v/v/v) had the same chromatographic mobility as an authentic standard sample of *N*-methylalanine in this and two other solvent systems. This purified sample gave a positive test for *N*-alkyl amino acids with *p*-nitrobenzoyl chloride–pyridine (Plattner and Nager, 1948) and for a secondary amine with nitroprusside–acetaldehyde (Feigl and Anger, 1937). The color produced by reaction with

ninhydrin is the same rare reddish brown seen with standard *N*-methylalanine and *N*-methylglycine (sarcosine) (Eloff and Grobbelaar, 1967). The infrared spectrum of this component from the acid hydrolysate was essentially the same as one for standard *N*-methylalanine. The nmr spectrum included the following assignments: a *C*-methyl doublet at δ 1.47 ppm, an *N*-methyl singlet at δ 2.70 ppm, and a methine proton multiplet at δ 3.61 ppm, all of which were identical with the nmr spectrum of standard *N*-methylalanine.

A purified sample of this compound was analyzed on a high-resolution mass spectrometer. The largest mass ion observed was at *m/e* 170 with an elemental composition of $C_8H_{14}O_2N_2$. The base peak that is assigned a 100% relative intensity value was at *m/e* 56 (C_3H_6N), another intense peak was at *m/e* 58 (C_3H_8N) with 79.2% relative intensity, and a third closely related peak was at *m/e* 57 (C_3H_7N) with a relative intensity value of 26.1%. The formula is suggestive of a cyclic dipeptide or diketopiperazine type structure. The mass spectrum was determined with a probe temperature of 350° and source temperature of 250°. At these high temperatures, the possibility of formation of the diketopiperazine of the amino acid seems good (Greenstein and Winitz, 1961). The formula for roseotoxin B, $C_{30}H_{49}O_7N_5$, cannot accommodate a structure whose elemental composition includes two oxygen and two nitrogen atoms in addition to the other known constituents.

If *N*-methylalanine (*m/e* 103 and $C_4H_9O_2N$) formed a diketopiperazine, the formula for that cyclic dipeptide would be the same as that reported for this component of the acid hydrolysate (*m/e* 170 and $C_8H_{14}O_2N_2$). This diketopiperazine would likely follow one of the fragmentation patterns for cyclodipeptides (Svec and Junk, 1964) and result in the formation of an "amine fragment," C_3H_7N with *m/e* 57, which then loses a proton to form the more stable fragment C_3H_6N with an *m/e* of 56. This C_3H_6N fragment is the same as the base peak in the mass spectrum of the compound that was analyzed. In view of this and other spectral and chromatographic information on this compound, we conclude that it is *N*-methylalanine.

trans-3-Methyl-L-proline. The only ninhydrin yellow component observed during tlc of the acid hydrolysate tested positive (blue color) for a secondary amino group with nitroprusside–acetaldehyde (Feigl and Anger, 1937). An infrared spectrum of the purified ninhydrin yellow component was essentially the same as one of the standard *trans*-3-methyl-L-proline. The nmr spectrum included the following: a triplet at δ 1.01 ppm, a doublet at δ 1.13 ppm, a singlet at δ 2.06 ppm, a multiplet at δ 3.48 ppm, and a singlet at δ 5.0 ppm.

This purified compound, which gives a yellow color with ninhydrin, was analyzed on a high-resolution mass spectrometer. The largest mass ion was at *m/e* 222, which had an elemental composition of $C_{12}H_{18}O_2N_2$. The mass spectrum was dominated by one major peak at *m/e* 84, the 100% relative intensity or base peak that had an elemental composition of $C_5H_{10}N$. The next most intense peaks were at masses *m/e* 69, 82, and 85 at 9.5, 6.0, and 6.0% relative intensities, respectively.

This empirical formula, $C_{12}H_{18}O_2N_2$, cannot be accommodated into that of the parent molecule, $C_{30}H_{49}O_7N_5$, in view of other known constituents. This formula is also suggestive of a diketopiperazine-type structure. The type of reaction in which a diketopiperazine forms does occur with proline compounds, usually peptides containing proline (Berger *et al.*, 1954). If methylproline (*m/e* 129 and $C_6H_{11}O_2N$) formed a diketopiperazine, the formula would be $C_{12}H_{18}O_2N_2$, which has a mol wt of 222. Once formed, the diketopiperazine would likely follow one of the fragmentation routes for cyclodipeptides (Svec and Junk, 1964) and result in the formation of an "amine fragment," $C_5H_{10}N$, with *m/e* of 84, which is identical with the mass spectral results obtained. The mass spectrum was determined with a spectrometer probe temperature of 150° and source temperature of 200°, temperature conditions under which diketopiperazines could form (Abderhalden and Hass, 1926). In view of the mass spectral data and other spectral and chromatographic data on this compound, we conclude that it is *trans*-3-methyl-L-proline. These samples sent for mass spectral analyses were probably not already in the diketopiperazine form before they were put into the mass spectrometer because both compounds are ninhydrin positive; diketopiperazines are ninhydrin negative (Johnson and Jackson, 1951).

N-Methylvaline. The ion-exchange chromatogram of an 8 *N* H_2SO_4 hydrolysate of roseotoxin B included a double peak with an elution time of 85–98 min (Figure 6). This raised the possibility of a fifth amino acid as part of the structure. This was not obvious from tlc of the acid hydrolysate. When the isoleucine fraction from tlc in butanol–acetic acid–water was developed twice in ethanol–water (90:10, v/v), the sample was resolved into two ninhydrin-positive components. The compound which had the smallest R_f gave a positive test for *N*-alkyl amino acids with *p*-nitrobenzoyl chloride–pyridine (Plattner and Nager, 1948) and for a secondary amine with nitroprusside–acetaldehyde (Feigl and Anger, 1937). The infrared spectrum of this component was essentially identical with the one for standard *N*-methylvaline. On the basis of this and other data (carbon-13 nmr spectra) we conclude that *N*-methylvaline is a constituent of the acid hydrolysate of roseotoxin B.

Hydroxy Acid. The retention times for several standard hydroxy acids and for the hydroxy acid in the acid hydrolysate of roseotoxin B were determined by gas chromatography as the free acid and as the methyl ester. Although retention times for standard β-hydroxy-*n*-butyric acid and the unknown hydroxy acid were the same (1.8 min), the retention times of their methyl esters were different, 1.8 and 0.5 min, respectively. Therefore, the unknown hydroxy acid was not β-hydroxy-*n*-butyric acid or any of the other 3-, 4-, 5-, or 6-carbon hydroxy acids tested.

Thin-layer chromatography of the same hydroxy acids on silica gel H plates with petroleum ether–diethyl ether–acetic acid (60:40:2, v/v/v) or chloroform–methanol–acetic acid (80:20:2, v/v/v) resolved several of the standard hydroxy acids from one another but has not provided definitive information about the unknown hydroxy acid.

The formula for the hydroxy acid which was determined by difference between the sum of the atoms of the other constituents and the parent molecule was $C_5H_8O_3$.

Double Bonds and Rings. Calculation of "double bonds and rings" as described by McLafferty (1967) for the formula of the hydroxy acid gave 2 degrees of unsaturation or the carbonyl and one other double bond. This explains our inability to obtain gas chromatographic identification of the unknown hydroxy acid since none of the standards contained a carbon–carbon double bond.

Calculation of "double bonds and rings" for the formula for roseotoxin B gave 9 degrees of unsaturation. The degrees of unsaturation are accounted for as follows: one for the carbonyl in each of the six hydrolysis products of the parent compound (6), and one each for the cyclic structure of the parent molecule, the ring in *trans*-3-methyl-L-proline, and the double bond in the side chain of the hydroxy acid.

Mass Spectrum of Roseotoxin B. Interpretation of the major ion fragments in Table I is as follows. The *m/e* 591 ion is the parent peak (P) and *m/e* 592 is the (P + 1) peak. The *m/e* 576 ion is 591 minus 15 or a CH_3 group, and the *m/e* 535 peak is 591 minus 56 or a C_4H_8 group from the isoleucine side chain. The *m/e* 534 ion is 591

Table I. Major Ion Fragments of Roseotoxin B between Mass 591 and 69[a]

m/e	Empirical formula	Rel intensity[b]
592	$C_{30}H_{50}O_7N_5$	0.9
591	$C_{30}H_{49}O_7N_5$	3.5
576	$C_{29}H_{46}O_7N_5$	14.3
535	$C_{26}H_{41}O_7N_5$	8.0
534	$C_{27}H_{42}O_7N_4$	26.0
506	$C_{25}H_{38}O_7N_4$	19.0
478	$C_{24}H_{38}O_6N_4$	4.7
449	$C_{23}H_{35}O_6N_3$	13.4
421	$C_{21}H_{31}O_6N_3$	15.6
182	$C_{10}H_{16}O_2N$	9.9
169	$C_9H_{15}O_2N$	6.6
168	$C_9H_{14}O_2N$	11.9
154	$C_9H_{16}ON$	19.9
86	$C_5H_{12}N$	100
85	$C_5H_{11}N$	7.5
84	$C_5H_{10}N$	64.9
69	C_4H_5O	7.9

[a] Fragments of intensity less than 5 have been omitted except those for which an assignment is made. [b] Percentage of base peak (*m/e* 86).

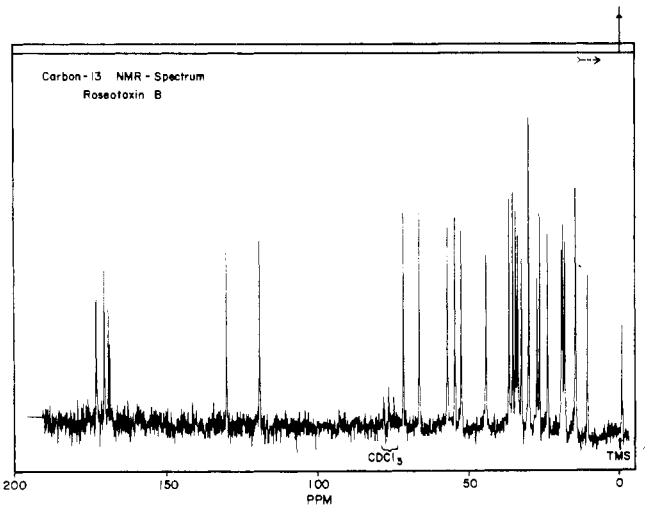

Figure 7. The carbon-13 proton decoupled nmr spectrum of roseotoxin B in $CDCl_3$. The chemical shifts (ppm) are measured from a tetramethylsilane (TMS) internal standard.

minus 57 or C_3H_7N (from *N*-methylalanine), and the *m/e* 506 ion is 591 minus 85 or $C_5H_{11}N$ from the isoleucyl or *N*-methylvalyl residue by the morpholine fragmentation route (Wulfson *et al.*, 1964). The *m/e* 478 ion is 591 minus 113 or $C_6H_{11}ON$ (isoleucyl or *N*-methylvalyl residue), and the 421 ion is 534 minus 113 or $C_6H_{11}ON$ as above.

Fragmentation patterns of cyclodepsipeptides have been studied by Wulfson *et al.* (1964, 1965). The most important fragmentation pattern for roseotoxin B, an irregular cyclohexadepsipeptide, is the morpholine type. Adjacent amino acids form 2,5-dioxomorpholine, and adjacent amino and hydroxy acids form 2,5-dioxopiperazine derivatives. Ion fragments at *m/e* 84, 85, and 86 form by this route and *m/e* 86 is the 100% relative abundance or base peak (Table I). The base peak forms from the isoleucine or *N*-methylvaline part of roseotoxin B; the fragment $C_5H_{11}N$ (*m/e* 85) then gains a proton for added stability to become $C_5H_{12}N$ (*m/e* 86). The next most abundant ion is $C_5H_{10}N$ (*m/e* 84) which can form from *trans*-3-methyl-L-proline (C_5H_9N) by gaining a proton for added stability or from isoleucine or *N*-methylvaline residues ($C_5H_{11}N$) by losing a proton. A second ion fragmentation pattern called "COX" begins with the elimination of CO_2, CONH, or $CONCH_3$ and results in the formation of peaks *m/e* 69, 168, 169, 182, and 449.

Table II. Carbon-13 Nmr Spectrum; Assignment of Lines

Line no.	Hydrogens directly bonded	ppm from TMS	Line assignment[e]	Lit. value
1	0	173.6	C=O (N-MeVal)	172.6[d]
2	0	173.6	C=O (Ile)	172.8[c]
3	0	171.1	C=O (N-MeAla)	173.8[b]
4	0	171.0	C=O (β-Ala)	
5	0	169.7	C=O (3-MePro)	
6	0	162.2	C=O (HO acid)	
7	1	131.0	γ-CH (HO acid)	
8	2	119.9	δ-CH_2 (HO acid)	
9	1	72.6	α-CH (HO acid)	
10	1	67.3	α-CH (3-MePro)*	
11	1	58.1	α-CH (Ile)	59.0[c]
12	1	55.6	α-CH (N-MeAla)	
13	1	53.5	α-CH (N-MeVal)	59.0[b]
14	2	45.2	δ-CH (3-MePro)*	
15	1	37.5	β-CH (Ile)*	37.9[c]
16	1	36.2	β-CH (3-MePro)*	
17	2	35.2	β-CH_2 (β-Ala)	38.4[a]
18	2	34.5	α-CH_2 (β-Ala)	35.7[a]
19	2	33.3	γ-CH_2 (3-MePro)	
20	2	30.8	γ-CH_2 (Ile)	26.0[c]
21	3	30.8	N-CH_3 (N-MeVal)	34.9[b]
22	3	28.1	N-CH_3 (N-MeAla)	34.9[d]
23	1	27.2	β-CH (N-MeVal)	27.0[b]
24	2	24.6	β-CH_2 (HO-Acid)	
25	3	20.0	β-CH_3 (3-MePro)	
26	3	19.6	γ-CH_3 (N-MeVal)	19.3[b]
27	3	18.9	γ-CH_3 (N-MeVal)	19.3[b]
28	3	15.3	γ-CH_3 (Ile)	17.0[c]
29	3	15.3	β-CH_3 (N-MeAla)	13.2[d]
30	3	11.3	δ-CH_3 (Ile)	12.7[c]

[a] Voelter *et al.* (1971). Values reported are for the free amino acid. [b] From unpublished results on actinomycin D. [c] Deslauriers *et al.*, (1972) and Voelter *et al.* (1971). [d] Voelter *et al.* (1971). Used values reported for alanine in *t*-BOC-Ala-Pro. These values were empirically corrected for incorporating an N-CH_3 on a peptide. [e] The confirmed assignments are indicated by asterisks.

Analysis of the Nuclear Magnetic Resonance Spectrum. (a) *Carbon-13 Nmr Spectrum.* The natural abundance carbon-13 nmr spectrum of roseotoxin B is presented in Figure 7 and a summary of the results is presented in Table II. There are 27 resolved absorption lines. In addition, scale expansion of the spectra clearly shows that the lines at 173.6 and 171.0 ppm are both overlapping lines. Finally intensity and line-width considerations indicate that the absorption at 30.8 ppm is also two transitions so the carbon count of 30 is confirmed. Single-frequency off-resonance decoupling was performed at two distinct decoupling frequencies to identify the number of directly attached protons associated with each carbon-13 resonance line (Table II). The number of protons in roseotoxin B was confirmed (49) and included 47 protons directly bonded to carbon plus two bonded to nitrogen. Of equal importance was the determination of the correct number of each type of carbon (methine, methylene, methyl, and carbonyl) for the proposed structure. The assignment of each carbon line to a specific carbon in roseotoxin B was initially made by comparison with values in the literature (Voelter *et al.*, 1971; Deslauriers *et al.*, 1972). Several of these assignments were confirmed by selectively decoupling individual protons in the partially assigned proton nmr spectrum.

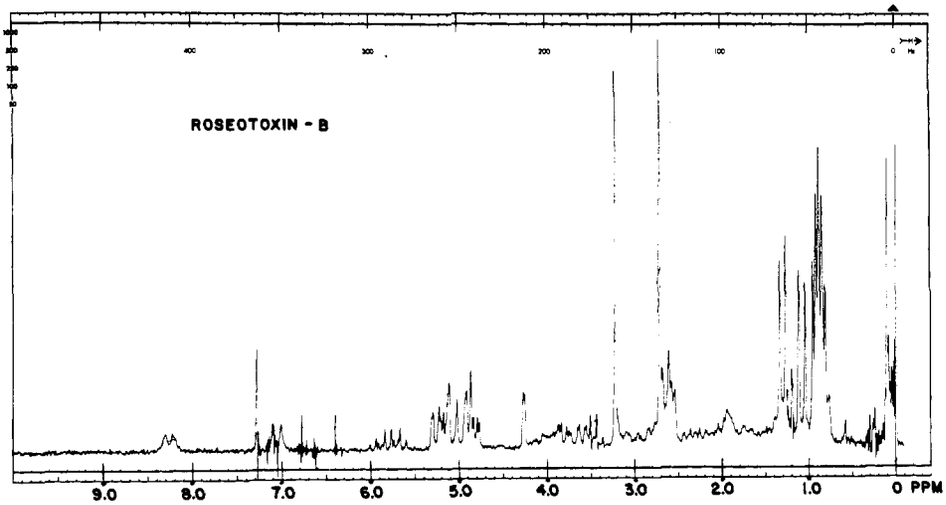

Figure 8. The 100-MHz proton nmr spectrum of rosetoxin B in $CDCl_3$ at 25°. The chemical shifts (ppm) are measured from a tetramethylsilane (TMS) internal standard.

(b) *Proton Spectrum.* The proton nmr spectrum of roseotoxin B is shown in Figure 8. Two N-H (7.1 and 8.3 ppm) and two *N*-methyl (2.7 and 3.2 ppm) resonances are easily identified from the spectrum and its integral. The remainder of the proton spectra was partially assigned by proton–proton double resonance techniques. A summary of the pertinent decoupling experiments follows. *Isoleucine.* The α-CH doublet at 4.85 ppm is coupled to the N-H proton at 7.1 ppm and to the β-CH at 2.0 ppm. Irradiation at 2.0 ppm simultaneously simplified the α-CH pattern and decouples the β-CH_3 doublet at 0.88 ppm.

N-Methylvaline. The α-CH doublet at 4.97 ppm has a characteristically large *J* (or coupling constant) of 11.0 Hz. This doublet collapses with irradiation at 2.3 ppm; simultaneously two methyl doublets decoupled to singlets. Also, irradiation of the α-CH doublet does not simplify any N-H patterns. Thus, the *N*-methyl structure of valine is supported.

N-Methylalanine. The α-CH quartet at 5.15 ppm collapses to a singlet upon irradiation of the methyl doublet at 1.3 ppm. Again, the absence of simplification of N-H patterns upon decoupling the α-CH indicates *N*-methyl.

β-Alanine. Double resonance indicates that the broad multiplet at 4.1 ppm is coupled to 3.1- and 2.3-ppm transitions as well as to the low-field N-H multiplet. Attempts to totally analyze these couplings were unsuccessful because of their complexity.

*trans-3-Methyl-*L*-proline.* The α-CH doublet at 4.26 ppm is coupled to resonances at 2.8 ppm. Irradiation at 2.8 ppm collapses the α-CH doublet as well as the methyl doublet at 1.1 ppm. The methyl group is thus definitely at the β-carbon or 3-methylproline. Further coupling between the nonequivalent γ- and δ-methylene protons was detected at 3.1, 2.0, and 3.55 ppm.

2-Hydroxy-4-pentenoic Acid. The α-CH triplet at 4.88 ppm decouples with irradiation at 2.62 ppm; at the same time the very complex vinyl pattern simplifies. The presence of a vinyl or olefin group was indicated in the carbon-13 spectrum by the two lines at 131.0 and 119.9 ppm. These data are consistent for the structure of 2-hydroxy-4-pentenoic acid.

Interpretation of carbon-13 and proton nmr spectra has confirmed the presence of one molecule of isoleucine, *N*-methylvaline, *trans*-3-methyl-L-proline, *N*-methylalanine, β-alanine, and 2-hydroxy-4-pentenoic acid per molecule of roseotoxin B. There was no conflicting nmr evidence for these proposed constituents in the structure of roseotoxin B.

In 1966, Suzuki *et al.* reported characterization of a toxic metabolite, destruxin A, from *Oospora destructor* which has the same acid hydrolysis products as roseotoxin B except one. Destruxin A, which was toxic to silkworms, contained proline instead of *trans*-3-methyl-L-proline. It appears that this is the first report of the occurrence of the imino acid, *trans*-3-methyl-L-proline as a part of the chemical structure of a mycotoxin.

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Metabolism of Carbaryl (1-Naphthyl *N*-Methylcarbamate) in Human Embryonic Lung Cell Cultures

Tsu H. Lin, Hanson H. North, and Robert E. Menzer*

Carbaryl (1-naphthyl *N*-methylcarbamate) was metabolized oxidatively by primary human embryonic lung cells in culture. After incubation of [^{14}C]carbaryl (300,000–400,000 dpm, about 3 μg) for 72 hr, 70% of the recovered radioactivity resulted from oxidative metabolites, with 30% being water soluble. The total recovery of added radioactivity was over 99%. Protein and nucleic acids determinations on cellular materials revealed that carbaryl at the applied dosage level did not inhibit cell growth and enzyme syntheses. Unconjugated metabolites from both cells and cell sonicates were identified as 1-naphthol, 5-hydroxycarbaryl, 4-hydroxycarbaryl, and 5,6-dihydro-5,6-dihydroxycarbaryl. The water-soluble materials released after HCl hydrolysis were identified as 4-hydroxycarbaryl, 1,4-naphthalenediol, and 5,6-dihydro-5,6-dihydroxycarbaryl. Several unknowns remained to be identified. The conjugated metabolites were possibly not *O*-glucuronides because β-glucuronidase treatment did not free aglycones from conjugation. Furthermore, the addition of UDPGA and other cofactors to the cell sonicates did not effect any increase in conjugations.

The metabolism of carbaryl (1-naphthyl *N*-methylcarbamate) has been studied extensively in plants (reviewed by Kuhr, 1970) and animals (reviewed by Dorough, 1970) by many investigators. Presently, however, only one paper on the metabolism of carbaryl in cell cultures has been published, indicating that investigators in insecticide toxicology and chemistry have just begun to use tissue or cell culture systems as a research tool. Cell cultures provide a means for investigating the direct action of pesticides on cells or tissues in the absence of the complex system of a whole organism (Rosenoer, 1966). Baron and Locke (1970) first published work on the metabolism of carbaryl in a cell culture system using an established cell line, L-132 human embryonic lung cells. They identified three aglycones of carbaryl as 1,4-naphthalenediol, 4-hydroxycarbaryl, and 5,6-dihydro-5,6-dihydroxycarbaryl; 1-naphthol was not found among the aglycones. These authors also suggested that the water-soluble metabolites were *N*-glucuronides instead of *O*-glucuronides because the water-soluble metabolites could not be hydrolyzed by β-glucuronidase. Many water-soluble metabolites remained unidentified.

The metabolic fate of carbaryl may vary with the age, stage of development, and type of cells. The present study reports the investigaton of carbaryl metabolism by primary human embryonic lung (HEL) cell cultures.

MATERIALS AND METHODS

Materials. [^{14}C]Carbaryl (1-[^{14}C]naphthyl *N*-methylcarbamate) was synthesized by reacting equimolar quantities of methyl isocyanate in toluene (1:2, v:v) and 1-[^{14}C]naphthol (20.8 mCi/mmol, Amersham/Searle Corp.) at 80° for 2 hr (Leeling and Casida, 1966). The product was identified by cochromatography with authentic standard carbaryl (Union Carbide Corporation) and had a specific activity of 12.16 mCi/mmol.

Several derivatives of carbaryl were also supplied by Union Carbide Corporation. Beef liver β-glucuronidase

Department of Entomology, University of Maryland, College Park, Maryland 20742.