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Amino Acid Sequence of Roseotoxin B

George W. Engstrom

The amino acid sequence of roseotoxin B, a toxic cyclodepsipeptide isolated from ether extracts of *Trichothecium roseum*, has been determined by isolation and purification of peptides from partial acid hydrolysates and by complete acid hydrolysis and hydrazinolysis of the purified peptides. The hydroxy and amino acid sequence for the compound is cyclo-2-hydroxy-4-pentenoyl-*trans*-3-methylprolyl-L-isoleucyl-*N*-methylvalyl-β-alanyl-*N*-methylalanyl.

In 1975, Engstrom et al. reported that the identity of the one hydroxy and five amino acids found in the acid hydrolysate of roseotoxin B were 2-hydroxy-4-pentenoic acid, *trans*-3-methylproline, L-isoleucine, *N*-methylvaline, β-alanine, and *N*-methylalanine. This new toxic cyclodepsipeptide isolated from *Trichothecium roseum* had a molecular weight of 591 and an empirical formula of $C_{30}H_{49}O_7N_5$. The degree and nature of the toxicity of roseotoxin B in ducklings and mice has been reported previously by Richard et al. (1969, 1970).

The term "cyclodepsipeptide" was coined by Shemyakin (1960) and refers to cyclic compounds with alternating amino and hydroxy acid residues such as enniatin A (Quitt et al., 1963; Audhya and Russell, 1976), amino acids only such as telomycin (Sheehan et al., 1968), and those that consist of amino acids plus one hydroxy acid such as isariin (Vining and Taber, 1962), and esperin (Ito and Ogawa, 1959). Tamura et al. (1964) and Suzuki et al. (1966, 1970) have published structures of five cyclodepsipeptides produced by culture filtrates of *Metarrhizium anisopliae* (Mitchnikoff) Sorokin (formerly called *Oospora destructor*) which is a fungus that is pathogenic for insects. These compounds, called "destruxins", are similar in amino acid composition to roseotoxin B but were different in the hydroxy acid, the proline derivative, the valine derivative, or a combination of these. Cyclodepsipeptides have been isolated from bacteria, actinomycetes, and fungi. Several reviews on cyclodepsipeptides and related compounds have been published by Schröder and Lübke (1963), Losse and Bachmann (1964a,b), Russell (1966), Taylor (1970), Andreev et al. (1972), and Pressman (1977).

In this paper, new data concerning the chemical characterization of roseotoxin B are reported. Purification of the peptides from partial acid hydrolysates and identification of their hydroxy and amino acid residues have permitted formulation of the sequence of hydroxy and amino acids in roseotoxin B.

EXPERIMENTAL SECTION

Materials. The sources of chemicals and supplies were as follows: ninhydrin, L-proline, L-isoleucine, *N*-methyl-DL-alanine, *N*-methyl-DL-valine, from Sigma Chemical Co., St. Louis, Mo., β-alanine from Calbiochem-Behring Corp., San Diego, Calif.; hydrazine, fluorescamine from Pierce Chemical, Rockford, Ill.; isatin and zinc acetate from Fisher Scientific, Fair Lawn, N.J.; *trans*-3-methylproline from Dr. A. G. Mauger, Washington Hospital Center, Washington, D.C., and silica gel H and silica gel G manufactured by E. Merck AG Darmstadt, Germany, and distributed by Brinkmann Instruments, Des Plaines, Ill.

Methods. The purification procedure of roseotoxin B was described previously by Engstrom et al. (1975). Partial acid hydrolysis experiments involved dissolving crystalline roseotoxin B in concentrated HCl at room temperature (21–23 °C). Periods of hydrolysis include 1 and 3.5 days after which excess hydrochloric acid was removed by repeated flash evaporation (Buchler Instr., Fort Lee, N.J.). The partial acid hydrolysates were dissolved in small volumes of water for application to 20 × 20 cm thin-layer chromatograph (TLC) plates coated with 0.375 mm layer of silica gel H. The plates were air-dried overnight and activated by heating to 110 °C for 1 h before they were used. All the solvents were analytical reagent or reagent grade. All partial acid hydrolysates were chromatographed in order to isolate and identify the portion of each fraction that contained a peptide.

The first step in purification was TLC on silica gel H with ethyl acetate as the solvent. Silica gel from the upper half of each TLC plate was transferred to a flask, eluted with water, filtered through Whatman No. 42 filter paper, and flash evaporated to dryness. This was designated fraction I and contained a product from the partial acid hydrolysate that did not react with ninhydrin. This compound was designated peptide I although one of the residues was shown to be a hydroxy acid. The silica gel from the lower half of each TLC plate was processed in the same way and contained all the other fractions. This sample was applied to silica gel H plates which were then developed in the solvent mixture of benzene–ethanol–concentrated ammonium hydroxide (57:114:37, v/v/v). The plates were air-dried, and a narrow vertical band was scribed on both edges of each plate, sprayed with ninhydrin (Fahmy et al., 1961), and placed in an oven at 100 °C for 20 min to develop the color. The color bands that developed were used as the basis for scribing horizontal lines

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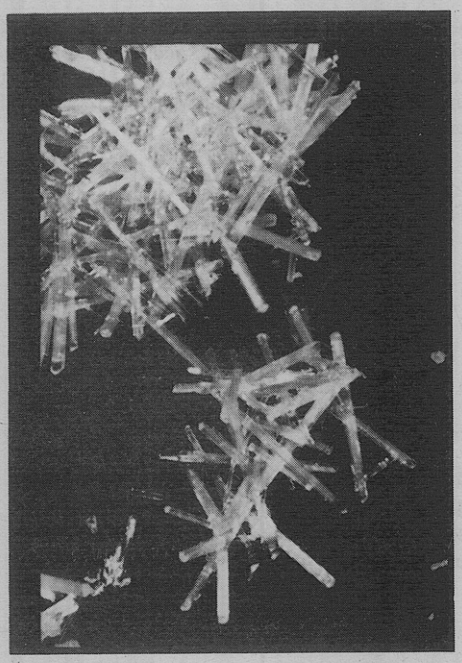


Figure 1. Roseotoxin B crystals from ether–petroleum ether.

to divide the sample into seven fractions numbered from the top of the plate to the origin. Peptides were found in fractions II, III, and VII and were used for sequence studies. Each fraction was scraped from the TLC plate, eluted with water, filtered through Whatman No. 42 filter paper, and flash evaporated to dryness.

All fractions were checked for homogeneity by TLC in three solvent systems. The solvent systems were benzene–ethanol–concentrated ammonium hydroxide (57:114:37, v/v/v), ethanol–water (88:12, v/v), and 1-butanol–glacial acetic acid–water (60:20:20, v/v/v). If a fraction gave more than one spot, it was rechromatographed in that system to homogeneity. A 2-mL aliquot of each purified peptide solution was transferred to glass vials together with 2 mL of concentrated HCl (final acid concentration of 5.7 N HCl), sealed, and heated in an autoclave to 120 °C for 24 h. These conditions were used to determine the complete hydroxy and/or amino acid composition of each peptide. The purified peptides and their acid hydrolysates were chromatographed in each of the three solvent systems, sprayed with ninhydrin, and heated to 100 °C for 20 min to develop the color. In similar experiments with the peptides, the TLC plates were sprayed with fluorescamine reagents (Udenfriend et al., 1972) or isatin–zinc acetate reagent (Barrolier et al., 1956).

Fluorescence of peptides that reacted with fluorescamine was observed with a long-wave ultraviolet lamp (Burton Ultraviolet Lamp, Model 19-0, Santa Monica, Calif.). The C-terminal amino acid of two of the peptides was determined by hydrazinolysis (Akabori et al., 1952; Niu and Fraenkel-Conrat, 1955). The N-terminal amino acid was determined by its reaction with fluorescamine, ninhydrin, and isatin–zinc acetate and the knowledge of which amino acids were present in the peptide from TLC of acid hydrolysates.

In thin-layer electrophoresis (TLE) experiments, equipment from Camag Inc., Milwaukee, Wis., was used. Thin-layer plates of silica gel G were sprayed with 0.05 M phosphate buffer (pH 6.0). This buffer solution was also used in the buffer troughs and to soak the filter paper conductor wicks. The TLE plates were allowed to air-dry in the horizontal position until the layer was no longer shiny, and then the samples were applied as spots. The voltage was set at 500 and current at 16 mA and the time for the TLE experiment was 105 min. The TLE plate was removed from the electrophoresis cell, dried in an oven at 110 °C for 1 h, cooled, and placed in an iodine jar or sprayed with ninhydrin and heated in an oven at 100 °C for 20 min to develop the color.

Roseotoxin B does not appear to cause any health hazard to man if reasonable care is exercised when working with it.

Table I. Thin-Layer Chromatography of Peptides from Roseotoxin B[a]

peptide	R_f in solvent system I[b]	II[c]	III[d]	IV[e]
roseotoxin B	0.28			
peptide I	0.71			
peptide II		0.81	0.37	0.68
peptide III		0.70	0.47	0.63
peptide IV		0.39	0.26	0.36

[a] The thin-layer chromatography plates consisted of a 0.375 mm thick layer of silica gel H on 20 × 20 or 5 × 20 cm glass plates. The plates were placed in an iodine jar or sprayed with ninhydrin. [b] Solvent system I was 100% ethyl acetate. [c] Solvent system II was benzene–ethanol–concentrated ammonium hydroxide (57:114:37, v/v/v). [d] Solvent system III was ethanol–water (88:12, v/v). [e] Solvent system IV was 1-butanol–glacial acetic acid–water (60:20:20, v/v/v).

Table II. Color Reactions for Distinguishing Peptides

peptide	ninhydrin[a]	isatin–zinc acetate[b]	fluorescamine[c]
peptide I	–	–	–
peptide II	+	+	–
peptide III	+	–	+
peptide IV	+	–	+

[a] Thin-layer chromatography plates sprayed with ninhydrin (Fahmy et al., 1961) were heated in an oven at 100 °C for 20 min to develop the color. [b] Thin-layer chromatography plates sprayed with isatin–zinc acetate (Barrolier et al., 1956) were heated in an oven at 80–85 °C for 30–60 min and stored in the dark for 24 h. [c] Thin-layer chromatography plates sprayed with 2.5% $NaHCO_3$ in water (pH 8.5), air-dried, and then sprayed with 30 mg % solution of fluorescamine (Udenfriend et al., 1972) in acetone and viewed under short-wave ultraviolet light.

RESULTS AND DISCUSSION

Partial acid hydrolysis of crystalline roseotoxin B (Figure 1) yielded four peptides that were isolated and purified by TLC. Each peptide contained two or three hydroxy and/or amino acid residues.

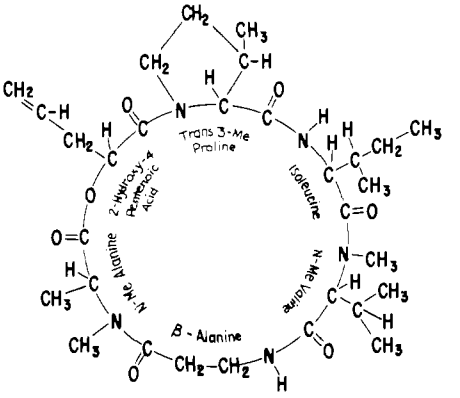
Peptide I was isolated and purified by TLC on silica gel H in ethyl acetate. This was the first step in purification of the peptides from the partial acid hydrolysates. It had an R_f of 0.71 compared to 0.28 for roseotoxin B (Table I) and picked up color in an iodine jar. It did not react with ninhydrin, isatin–zinc acetate, or fluorescamine (Table II). The peptide was then submitted to treatment with 5.7 N HCl in a sealed glass vial in an autoclave at 120 °C for 24 h to determine the number and identity of hydroxy and/or amino acid residues. The hydrolysate was chromatographed in ethanol–water, (88:12, v/v) and 1-butanol–glacial acetic acid–water (60:20:20, v/v/v). The TLC plates were sprayed with ninhydrin and heated to 100 °C for 20 min to develop the color. One spot appeared, and it had the same R_f as standard *trans*-3-methylproline in the three solvent systems. The spot was yellow.

trans-3-Methylproline was the only residue of those previously reported in roseotoxin B (Engstrom et al., 1975) that gave a yellow color in reaction with ninhydrin. The fact that peptide I did not react with ninhydrin showed that there was not a free amino or imino functional group available for that reaction. After hydrolysis, one functional group reacted with ninhydrin to give the yellow color. This peptide must have the one hydroxy acid in roseotoxin B protecting the imino group of an imino acid residue. Therefore the sequence of peptide I was 2-hydroxy-4-pentenoyl-*trans*-3-methylproline.

Peptide II was homogeneous by TLC in ethanol–water (88:12, v/v); 1-butanol–glacial acetic acid–water (60:20:20, v/v/v); and benzene–ethanol–concentrated ammonium hydroxide (57:114:37, v/v/v). The R_f values are listed in Table I. This peptide was purified from fraction III of the partial acid hydrolysate. The color produced with ninhydrin went through an unusual color transition from pale yellow through burnt orange to a pink red. The acid hydrolysate of peptide II contained three amino acid residues that were resolved by TLC in ethanol–water (88:12, v/v) and benzene–ethanol–concentrated ammonium hydroxide (57:114:37, v/v/v). The R_f of the spots were the same as those for standard L-isoleucine, *N*-methyl-DL-valine, and *trans*-3-methylproline in the three solvent systems. The fact that the color development of peptide II with ninhydrin included a yellow color (transitional) and a final pink red that fluoresced red under long-wave ultraviolet light (Parmentier and Vanderhaeghe, 1960; Piez et al., 1956) was evidence for *trans*-3-methylproline being the N-terminal amino acid of peptide II. This peptide also gave a positive test (blue color) with the isatin–zinc acetate reagent which is a test for proline and certain proline derivatives (Table II). The fact that peptide II did not react with fluorescamine showed that the N-terminal amino acid did not have a primary amino group available for this reaction (Table II). Hydrazinolysis of peptide II gave one spot after TLC that had the same R_f as standard *N*-methyl-DL-valine in each of the three solvent systems. This proved that *N*-methylvaline was the C-terminal amino acid of the peptide II. The amino acid sequence of peptide II was *trans*-3-methylprolyl-L-isoleucyl-*N*-methylvaline.

Peptide III was homogeneous by TLC in ethanol–water (88:12, v/v), 1-butanol–glacial acetic acid–water (60:20:20, v/v/v), and benzene–ethanol–concentrated ammonium hydroxide (57:114:37, v/v/v). The R_f values are listed in Table I. This peptide was purified from fraction II of the partial acid hydrolysate. The acid hydrolysate of peptide III contained two amino acid residues that were resolved by TLC in ethanol–water (88:12, v/v). The R_f of the spots were the same as those for standard L-isoleucine and *N*-methyl-DL-valine in the three solvent systems. Peptide III reacted with fluorescamine to give a strong fluorescent spot on the TLC plate (Table II). The primary amino group of isoleucine can react with fluorescamine but *N*-methylvaline does not have that functional group and does not react under the conditions of the assay (Udenfriend et al., 1972) (Table II). The sequence of amino acids in peptide III was L-isoleucyl-*N*-methylvaline.

Peptide IV was homogeneous by TLC in ethanol–water (88:12, v/v), 1-butanol–glacial acetic acid–water (60:20:20, v/v/v) and in benzene–ethanol–concentrated ammonium hydroxide (57:114:37, v/v/v). The R_f values are listed in Table I. This peptide was purified from fraction VII of the partial acid hydrolysate. The acid hydrolysate of peptide IV contained two amino acid residues that were resolved by TLC in benzene–ethanol–concentrated ammonium hydroxide (57:114:37, v/v/v). The two ninhydrin positive spots from peptide IV had the same R_f values as those for standard β-alanine and *N*-methyl-DL-alanine. Peptide IV reacted with fluorescamine to give a strong fluorescent spot on the TLC plate (Table II). β-Alanine has a primary amino group that can react with fluorescamine but *N*-methylalanine does not and so cannot react under the conditions of the assay (Udenfriend et al., 1972). Thin-layer chromatography of the hydrazinolysate of peptide IV gave one spot which reacted with ninhydrin and it had the same R_f value as standard *N*-methyl-DL-alanine in each of the three solvent systems. This proved that the C-terminal amino acid of peptide IV was *N*-methylalanine and the sequence was β-alanyl-*N*-methylalanine.



ROSEOTOXIN B

Figure 2. Amino acid sequence and structure of roseotoxin B.

THIN-LAYER ELECTROPHORESIS

Thin-layer electrophoresis experiments with roseotoxin B showed that its migration distance with 0.05 M phosphate buffer (pH 6.0) was only 0.5 cm toward the cathode whereas isoleucine was 1.7, histidine 7.1, lysine 7.2, and arginine 7.4 cm. Aspartic acid moved 5.2 cm toward the anode. This observation was consistent with the report by Russell (1966) in which he stated that natural cyclodepsipeptides are usually neutral compounds.

CONCLUSIONS

In 1966, Suzuki et al. reported characterization of a toxic metabolite, destruxin A, which has the same acid hydrolysis products as roseotoxin B with one exception. Destruxin A contained proline instead of *trans*-3-methylproline. The amino acid sequence of destruxin A was similar to what we have found for roseotoxin B. The difference was found in the order of the last two amino acids in the sequence.

The sequence of hydroxy and amino acids in roseotoxin B has been established by determining the sequences of the four peptides isolated from the partial acid hydrolysates and by fitting them together to give the complete sequence. The sequence of residues in the four peptides were as follows: peptide I, 2-hydroxy-4-pentenoyl-*trans*-3-methylproline; peptide II, *trans*-3-methylprolyl-L-isoleucyl-*N*-methylvaline; peptide III, L-isoleucyl-*N*-methylvaline; peptide IV, β-alanyl-*N*-methylalanine.

The complete sequence of hydroxy and amino acids for roseotoxin B is proposed to be cyclo-2-hydroxy-4-pentenoyl-*trans*-3-methylprolyl-L-isoleucyl-*N*-methylvalyl-β-alanyl-*N*-methylalanyl. A diagrammatic presentation of the structure is shown in Figure 2.

Certain aspects of the structure remain to be determined by future work. The stereochemical configuration of *trans*-3-methylproline, *N*-methylalanine, *N*-methylvaline,

and 2-hydroxy-4-pentenoic acid need to be determined. Furthermore, in the absence of a peptide with a sequence overlapping the β-alanine residue, inversion of the sequence during partial acid hydrolysis is a possibility, although an unlikely one. Therefore the sequence of this part of the molecule is not yet unequivocal. One approach to this problem would be to isolate the hydroxyl acyl peptide resulting from alkaline hydrolysis of roseotoxin B (Engstrom et al., 1975) and determine the C-terminal amino acid.

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Peroxide Oxidation Products of Homocystine and Lanthionine

Samuel H. Lipton

Isolation of DL-homocysteic acid, DL-homolanthionine sulfone, and DL-homolanthionine as crystalline products of peroxide oxidation of DL-homocystine in HCl solution confirmed structures deduced from amino acid analysis. DL-Homocysteic acid, produced in 4.8 N HCl, was easily isolated because it was not adsorbed by Dowex-50-H^+ resin. Similar oxidation in 0.8 N HCl produced DL-homolanthionine sulfoxide. This sulfoxide intermediate was heated in 6 N HCl to form DL-homolanthionine sulfone which crystallized from NH_4OH solution. Reduction of the sulfoxide by dimethyl sulfide in 6 N HCl formed DL-homolanthionine which crystallized from NH_4OH solution following ion-exchange chromatography. This is a simple new preparation of DL-homolanthionine from inexpensive DL-homocystine. *S*-Methyl-L-cysteine and *S*-ethyl-L-cysteine were oxidized by peroxide in 0.8 N HCl to sulfones. Thus these thioethers resembled methionine in yielding sulfones, unlike *meso*-lanthionine which mainly yielded sulfoxides in 0.8 N HCl. However, the main oxidation product of *meso*-lanthionine in 4.8 N HCl solution was the sulfone which crystallized from NH_4OH solution.

Peroxide oxidation of the sulfur amino acids cystine, lanthionine, and homocystine formed products that varied with reaction conditions, particularly hydrochloric acid concentration (Lipton et al., 1977). An especially interesting feature of the peroxide oxidation of the disulfide amino acids was the loss of 1 mol of sulfur. The formation of homolanthionine sulfoxide and sulfone by peroxide oxidation of homocystine was first reported by Clopath and McCully (1976). Homocystine oxidation was further studied and the analogous formation of lanthionine sulfoxide and sulfone from peroxide oxidation of cystine was reported (Lipton et al., 1977). These chemical losses of sulfur from disulfide amino acids are of interest because lanthionine is formed in heated proteins (Hurrell et al., 1976). In the present study, peroxide oxidations were on a larger scale so that products could be purified and the assigned structures (Lipton et al., 1977) could be confirmed. DL-Homocysteic acid, DL-homolanthionine sulfone,

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