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Structure of Iturine A, a Peptidolipid Antibiotic from *Bacillus subtilis*†

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ABSTRACT: A mixture of iturines extracted from *Bacillus subtilis* gave, on column chromatography, iturine A, iturine B, and iturine C. Iturine A has the entire antifungal activity. It is a mixture of two homologous peptidolipids $C_{48}H_{74}N_{12}O_{14}$ and $C_{49}H_{76}N_{12}O_{14}$ (mp 177 °C, $[\alpha]_D$ −1.7° in methanol (*c* 0.05 g/mL); mol wt 1042 and 1056). The lipid moiety is a mixture of 3-amino-12-methyltridecanoic acid and 3-amino-12-methyltetradecanoic acid. The peptide moiety contains 7 mol of amino acids: D-Asn_2, L-Asn, L-Gln, L-Pro, L-Ser, and D-Tyr. A cyclic structure for iturine A with the serine residue linked to the fatty amino acids through a peptide bond has been demonstrated. By mild HCl hydrolysis, lipid-soluble and water-soluble peptides were obtained. They were analyzed by chemical methods and by mass spectrometry. Permethylated and perdeuteriomethylated derivatives of iturine A were also subjected to mass spectrometric analysis. Both chemical analysis and mass spectrometry led to the cyclic structure I for iturine A.

A polypeptide antibiotic named iturine was isolated from a culture medium of a strain of *Bacillus subtilis* collected in Ituri, Belgian Congo (presently Zaire). It has a high antifungal activity against various strains of yeasts and fungi (Delcambe & Devignat, 1957). The purification and characteristics of this antibiotic were previously described (Delcambe, 1965). A reinvestigation of iturine revealed it to be a mixture of iturine A, iturine B, and iturine C which were separated by column chromatography. Iturine A is the major constituent (30% of the crude iturine) having the entire antifungal activity; iturine B (5%) and iturine C (10%) have no antifungal activity. The lipid part of iturine A was shown to be a mixture of two long-chain β-amino acids (Peypoux et al., 1973). The present paper reports the complete structural determination of iturine A.

Materials and Methods

Solvent systems used for chromatography are: (A) chloroform–methanol–acetic acid (92:5:3); (B) chloroform–methanol–pyridine–water (40:14.5:14:2.5); (C) phenol–10% sodium citrate (1:1); (D) butanol–acetic acid–water (65:10:25); (E) chloroform–methanol–water (65:25:4); (F) pyridine–*tert*-amyl alcohol–water (35:35:30); (G) butanol–acetic acid–water (5:1:2); (H) benzene–pyridine–acetic acid (20:5:1); (I) benzene–pyridine–formic acid–acetic acid (75:25:1:0.2); (J) propanol–34% ammonia (7:3).

Amino Acid Analysis. Total hydrolyses of iturine A and lipopeptides with 6 N HCl were carried out at 150 °C for 8 h and similar hydrolyses of water-soluble peptides were performed at 105 °C for 18 h. The quantitative analysis of amino acids (ca. 150–200 nmol) was performed on a Technicon amino acid analyzer following the procedure of Spackman et al. (1958) modified by Piez & Morris (1960). A different procedure described by Ghuysen et al. (1966) was also used with smaller quantities of amino acids (ca. 50 nmol): the amino acids were dinitrophenylated, and the DNP derivatives were separated by thin-layer chromatography in solvents A or B, collected from the silica gel, and quantitatively estimated by spectrophotometry at 360 nm.

Configuration of amino acids was determined enzymatically on 25 nmol of each amino acid. Pro, Ser, and Tyr were incubated for 2 h at 37 °C with D-amino acid oxidase (Worthington; 170 μg) in a final volume of 50 μL of 0.1 M $K_4P_2O_7$ (pH 8.3) (Ishii & Witkop, 1963). Glu was incubated for 2 h at 37 °C in the presence of L-glutamate decarboxylase (Sigma) (10 μg) in a final volume of 40 μL of 0.2 M $NaCOOCH_3$ (pH 4.5; Gale, 1965). After enzymatic digestion, DNP derivatives of residual amino acids were estimated by spectrophotometry. The optical configuration of Asp was determined with L-glutamate–oxalacetate transaminase (Sigma; 20 μg) by incubation in a sodium arsenate buffer, 0.05 M, pH 7.6 (35 μL) with α-ketoglutarate (50 nmol). DNP derivatives of residual Asp and released Glu were estimated by spectrophotometry.

Identification of Asparaginyl and Glutaminyl Residues. The method of Ressler & Kashelikar (1966) was used. A solution of iturine A (6 mg) in triethyl phosphite (0.5 mL) was added to ethylene chlorophosphite (0.2 mL) at 100 °C for 18 h. After elimination of the excess reagent and solvent, the

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product was treated with sodium in liquid ammonia. Ammonia was evaporated and the water-soluble material was desalted by adsorption on a column of Dowex 50-X2 H^+ (5 × 0.8 cm) and subsequent elution with 30 mL of 3 N aqueous ammonia. After 6 N HCl hydrolysis at 110 °C for 18 h, the amino acids were analyzed by two-dimensional thin-layer chromatography on cellulose-gel H (9:5) (Turner & Redgwell, 1966) in the solvent systems C and D and also by Technicon autoanalyzer.

Partial Hydrolysis and Isolation of Peptides. Iturine A (50 mg) was hydrolyzed by 6 N HCl (6 mL) at 105 °C for 16 h. The lipid-soluble products (14 mg) were extracted with chloroform, analyzed by thin-layer chromatography on silica gel with solvent E, and separated by preparative thin-layer chromatography in the same solvent. Other lipid-soluble products were obtained from 10 N HCl hydrolyzate (120 mg of iturine A in 15 mL of HCl) at 80 °C for 3 h. No water-soluble peptide was present in this hydrolyzate which was fractionated by chromatography on a column of 40 g of silicic acid Bio-Sil HA 325 mesh (Bio-Rad Laboratories) and elution with solvent E. Further fractionation and purification were performed by thin-layer chromatography on a mixture of DEAE-cellulose–silica gel H (9:8) in the solvent E.

Water-soluble peptides resulted from the hydrolysis of iturine A (8 mg) in 3 N HCl (2 mL) at 105 °C for 4 h or from 0.1 N HCl (4 mL) hydrolysis of iturine A (40 mg) at 105 °C for 6 h. The water-soluble peptides were prepared by chromatography on Whatman no. 1 paper in the solvent F; lipophilic products migrated with the solvent front. Some peptides were further purified by a second paper chromatography in the solvent G.

Structural Determination of Peptides. N-terminal sequence of peptides was determined by Edman degradation (50 nmol of peptide for each cycle) according to Gray & Smith (1970). The N-terminal amino acids of water-soluble peptides were determined as dansyl derivatives by thin-layer chromatography on silica gel using the solvents H and I (Stehelin & Duranton, 1969). N-terminal amino acids of lipid-soluble peptides were identified as dinitrophenyl derivatives following the procedure of Ghuysen et al. (1968). C-terminal groups were determined on ca. 150 nmol of peptides by hydrazinolysis according to Ghuysen et al. (1968).

Paper Electrophoresis. These were carried out on Whatman no. 1 paper at pH 3.9 in pyridine–acetic acid–water (7.5:25:1000) and at pH 8.2 in 60 mM Veronal buffer at 40 V/cm using a Pherograph apparatus.

Alkalimetric Titration. Iturine A (1 mg) dissolved in 0.1 M NaCl (1 mL) was titrated with a M/160 solution of NaOH in nitrogen atmosphere using a Radiometer apparatus (Copenhagen).

Molecular Weight Determination. The molecular weight of iturine A was determined by a thermoosmotic method according to Brady et al. (1951) using a Mechrolab apparatus with thermistors (Muller & Stolten, 1953). A solution of iturine A (20 mg) in ethanol (1 mL) was used for this determination. The precision of the measurement was within ±3% (by comparing with standard samples).

Chromic Acid Oxidation. Iturine A (2 mg) was added to a solution of chromic anhydride (10 mg) in acetic acid–pyridine (30:1; 0.3 mL; Sheehan et al., 1958). The reaction was carried out at 25 °C for 20 h and the products were hydrolyzed with 6 N HCl (0.1 mL) at 150 °C for 8 h. The hydrolyzate was desalted on a column of Dowex 2-X8 (5 × 0.8 cm) and analyzed by paper chromatography in solvent F.

Acetylation, Permethylation, and Mass Spectrometry. Peptide P_4 (1 μmol) from partial hydrolysis of iturine A was N-acetylated with an equimolecular mixture (15 μL) of acetic anhydride and deuterated acetic anhydride in methanol (50 μL). After standing at room temperature for 1 h, the solvent was removed in a stream of nitrogen. Permethylation of N-acetylated peptide P_4 as well as of iturine A (0.5 mg) was effected with methyl iodide (200 μL) or trideuteriomethyl iodide (CEA, Saclay, France) in the presence of a solution (100 μL) of sodium hydride (Fluka; 50 mg in 50% oil dispersion, washed three times with dry ether) dissolved in dimethyl sulfoxide (2 ML; Hakomori, 1964); mass spectra were obtained with an AEI MS9 instrument operating at 70 eV, using a source temperature ranging from 200 to 270 °C.

Results and Discussion

Isolation and Properties of Iturine A. Crude iturine (20 g per batch) was prepared at the Centre National de Production et d'Etude des Substances d'Origine Microbienne (Liège, Belgium) in batches of 500 L of culture medium under conditions previously described (Delcambe, 1965). Purification (in fractions of 1 g of crude iturine) by chromatography on a Bio-Sil HA column (80 × 1.5 cm) with solvent E (elution volume of iturine A: 200 mL) provided iturine A (300 mg), a white powder [mp 177 °C; $[\alpha]_D$ −1.7° in methanol (*c* 0.05 g/mL); R_f 0.49 in the solvent system E] readily soluble in methanol and dimethylformamide, slightly soluble in water and ethanol, and insoluble in chloroform, ethyl ether, ethyl acetate, acetone, pyridine, and tetrahydrofuran. The UV spectrum in ethanol shows two maxima at 203 nm (ε 37 700) and 277 nm (ε 1810). The IR spectrum has peaks at 3.1, 6, and 6.5 μ. The molecular weight by osmometry was found to be 1055 ± 32. The antifungal activity tested on *Penicillium chrysogenum* gave a complete growth inhibition at a concentration of 5 μg per mL of medium.

Amino Acid Composition. After total hydrolysis of iturine A (6 N HCl, 150 °C, 8 h), water-soluble and lipid-soluble amino acids were obtained. The lipid-soluble amino acids (20% of iturine A) are a mixture of 3-amino-12-methyltridecanoic acid (C_{14} β-amino acid; 40%) and of 3-amino-12-methyltetradecanoic acid (C_{15} β-amino acid; 60%; Peypoux et al., 1973). Quantitative analysis of the water-soluble amino acids (from 200 nmol of iturine A) gave the molar ratio: $Asp_{3.2}$, Glu_1, $Pro_{0.9}$, $Ser_{0.9}$, $Tyr_{0.8}$ (Asp_3, Glu, Pro, Ser, Tyr). The optical configuration of the amino acids was determined by enzymatic methods (see Materials and Methods). Glu, Pro, Ser, and 1 mol of Asp per mol of iturine A have L configuration. Tyr and 2 mol of Asp have D configuration.

By paper electrophoresis at pH 3.9 or at pH 8.2, iturine A was shown to be neutral and an alkalimetric titration indicated the absence of carboxyl groups. On the other hand, the Technicon analysis of a total hydrolyzate of iturine A showed an intense peak of ammonia. These results are compatible with the presence of amidated groups in the dicarboxylic amino acids. The presence of Asn and Gln in iturine A was therefore tested by formation of α,ω-diamino acids from Asn and Gln following the procedure of Ressler & Kashelikar (1966). Dehydration and reduction of iturine A were performed (see Materials and Methods) and the products of the reaction were hydrolyzed, desalted, and analyzed by thin-layer chromatography and by autoanalysis on a Technicon apparatus. Asp and Glu were no longer present in the hydrolyzate, while Dab and Orn were found in the molar ratio 3:1. Consequently, Asn and Gln are present in iturine A in the molar ratio 3:1. The percentage of lipid-soluble amino acids and the molecular weight of iturine A indicate the presence of 1 mol of C_{14} or C_{15} β-amino acid per mol of iturine A. This result was confirmed by mass spectrometry (see later). Hence iturine A is composed

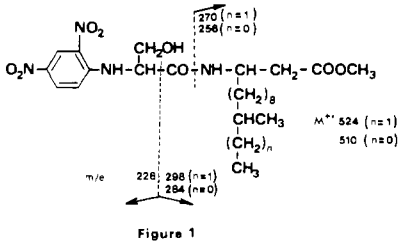


FIGURE 1: Principal fragmentation observed in the mass spectrum of dinitrophenyl methyl ester of the dipeptide Ser → C_{14},C_{15} β-amino acids.

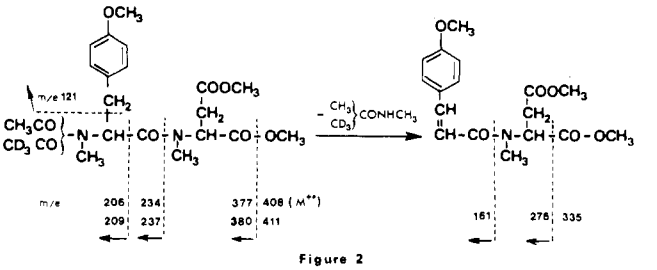


FIGURE 2: Principal fragmentation observed in the mass spectrum of *N*-acetyl (CH_3CO + CD_3CO) permethyl derivative of peptide P_4.

of: (βNC_{14},C_{15}),[1] D-Asn_2, L-Asn, L-Gln, L-Pro, L-Ser, and D-Tyr.

Presence of O-Unsubstituted Tyr and Ser. Iutrine A (2 mg) was treated with dinitrofluorobenzene. After total hydrolysis, no DNP derivative was extracted with ether. In the aqueous phase a DNP derivative was found and identified as *O*-DNP Tyr by thin-layer chromatography on silica gel in solvent J (Brenner et al., 1961). This indicates that the phenol group of Tyr is free and also establishes the absence of an N-terminal amino group in iturine A.

The infrared spectrum of iturine A lacks an ester absorption, thereby suggesting that the hydroxyl group of serine is free. A confirmation of this was obtained by chromic acid oxidation of iturine A followed by hydrolysis. Absence of Ser and Tyr in the hydrolyzate confirms that the hydroxyl groups of these two amino acids in iturine A are not substituted. This result as well as the absence of free carboxyl and amino groups can be explained on the basis of a cyclic structure for iturine A. The calculated molecular weights corresponding to such a structure (mol wt 1042 for βNC_{14} homologue and 1056 for βNC_{15} homologue) are in good agreement with the experimental value (mol wt 1055 ± 32). The cyclic structure was confirmed by mass spectrometry (see later).

Determination of the Lipid–Peptide Linkage. The chloroform extract of the 6 N HCl hydrolyzate at 105 °C for 16 h represented 28% of iturine A (see Materials and Methods), whereas it was only 20% after a total hydrolysis with 6 N HCl at 150 °C for 8 h (Peypoux et al., 1973). Besides the C_{14},C_{15} β-amino acids, another lipid-soluble fraction was isolated from the 105 °C hydrolyzate by thin-layer chromatography on silica gel in solvent E. Further hydrolysis of this fraction with 6 N HCl at 150 °C for 8 h gave Ser and the C_{14},C_{15} β-amino acids. Evidence of a peptide bond between Ser and the lipid part of iturine A rests upon the following observations: (1) the OH of Ser was found to be free in the molecule after chromic oxidation; (2) resistance to hydrolysis of this peptide bond is in accord with an N-terminal position for the Ser residue (Harris et al., 1956); (3) dinitrophenylation followed by acid hydrolysis gave DNP-Ser identified by thin-layer chromatography on silica gel in solvents A, B, and E; (4) after one Edman degradation, Ser was eliminated and released lipid-soluble products were identified as the C_{14},C_{15} β-amino acids by thin-layer chromatography (R_f 0.63) on silica gel in solvent E. The Ser-C_{14},C_{15} β-amino acid structure was further confirmed by mass spectrometry of *N*-dinitrophenyl methyl esters. Mass spectrum exhibited weak homologous molecular ion peaks differing by 14 mass units at *m/e* 524 and 510. Other homologous peaks observed at *m/e* 298, 284 and 270, 256 as well as a peak at *m/e* 226 corresponding to cleavages shown in Figure 1 are consistent with the proposed structures for the two homologous dipeptides Ser-C_{14} β-amino acid and Ser-C_{15} β-amino acid.

Peptide Sequence. The peptide sequence followed from a study of the water-soluble and lipid-soluble peptides obtained by partial hydrolysis of iturine A (see Materials and Methods).

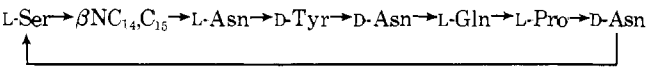
Lipid-Soluble Peptides. Chromatography of a 10 N HCl hydrolyzate (120 mg of iturine A at 80 °C for 3 h) on silicic acid Bio-Sil HA with solvent E gave two lipid-soluble fractions A (65 mg) and B (30 mg).

Further fractionation of A by preparative thin-layer chromatography on DEAE-cellulose–gel H (9:8) in solvent E led to the isolation of two products A_1 (30 mg, R_f 0.43) and A_2 (10 mg, R_f 0.67).

Fraction B (30 mg) was further fractionated by thin-layer chromatography on silica gel H in solvent E and gave two distinct products B_1 (12 mg, R_f 0.40) and B_2 (8 mg, R_f 0.46).

Water-Soluble Peptides. They were obtained by preparative paper chromatography of partial hydrolyzates of iturine A (see Materials and Methods). Four peptides P_1 (1 μmol), P_2 (1.5 μmol), P_3 (1.2 μmol), and P_4 (2 μmol) were isolated.

All lipid-soluble and water-soluble peptides mentioned above were shown pure; they gave a single spot by thin-layer or paper chromatography in various solvents followed by detection with ninhydrin reagent (Russell, 1960) and Pauly reagent (see Table I). Table I presents the quantitation of amino acids, their optical configuration, and the determination of N- and C-terminal groups. Quantitative analyses were done either with a Technicon autoanalyzer or by quantitative estimation of DNP derivatives as described in Materials and Methods. In addition to α-amino acids, lipid-soluble peptides A_1, A_2, B_1, and B_2 contain 1 mol of C_{14},C_{15} β-amino acid per mol of peptide. Configuration of α-amino acids and structural determination of peptides were established as described in Materials and Methods. The structure of P_4 was also confirmed by mass spectrometry of its *N*-acetyl permethylated derivative. The significant peaks in the mass spectrum correspond to fragment ions shown in Figure 2 and confirm the sequence Tyr → Asp for the dipeptide P_4. On the basis of the above data, the complete amino acid sequence in iturine A can be represented by the following cyclic structure:



Mass Spectrometry of Iturine A Derivatives. Mass spectrometry (Das & Lederer, 1971) of permethylated and perdeuteriomethylated iturine A fully confirms the above structure. The mass spectrum (Figure 3) of permethylated iturine A shows the two homologous molecular ion peaks at *m/e* 1280 and 1294 which are shifted to *m/e* 1331 and 1345, respectively, in the mass spectrum of perdeuteriomethylated iturine A. The

[1] Abbreviation used: βNC_{14},C_{15}, mixture of two homologous C_{14},C_{15} β-amino acids.

TABLE I: Characteristics, Chemical Composition, and Terminal Amino Acids of Lipid-Soluble and Water-Soluble Peptides.

peptide	hydrolysis conditions	R_f	molar ratios of α-amino acids (mol/mol of peptide) L-Asp	D-Asp	L-Glu	L-Pro	L-Ser	D-Tyr	N-terminal amino acid original peptide	*af*	*g*	C term. A A	peptide structure
A_1	10 N HCl, 80 °C, 3 h	0.43[b] 0.63[c]	1.0				0.8	0.8	L-Ser	βN C_{14},C_{15}		D-Tyr	L-Ser → βN C_{14},C_{15} → L-Asp→ D-Tyr
A_2	10 N HCl, 80 °C, 3 h	0.67[b] 0.63[c]	0.9	1.0			0.8	0.8	L-Ser	βN C_{14},C_{15}		D-Asp	L-Ser → βN C_{14},C_{15} → (L-Asp, D-Tyr) → D-Asp
B_1	10 N HCl, 80 °C, 3 h	0.27[b]											
	6 N HCl, 105 °C, 16 h	0.40[c]							L-Ser	βN C_{14},C_{15}			L-Ser → βN C_{14},C_{15}
B_2	10 N HCl, 80 °C, 3 h	0.46[c]	1				0.8		L-Ser	βN C_{14},C_{15}		L-Asp	L-Ser → βN C_{14},C_{15} → L-Asp
P_1	3 N HCl, 105 °C, 4 h	0.04[d] 0.16[e]		1	1				D-Asp	L-Glu		L-Glu	D-Asp → L-Glu
P_2	3 N HCl, 105 °C, 4 h	0.08[d] 0.18[e]		1	0.8	1			L-Glu	L-Pro	D-Asp	D-Asp	L-Glu → L-Pro → D-Asp
P_3	0.1 N HCl, 105 °C, 6 h	0.18[d] 0.37[e]			1	1			L-Glu	L-Pro			L-Glu → L-Pro
P_4	0.1 N HCl, 105 °C, 6 h	0.31[d] 0.46[e]		1				0.8	D-Tyr	D-Tyr			D-Tyr → D-Asp

[a] Presence of an N-terminal β-amino acid does not permit further degradation. [b] R_f values by thin-layer chromatography on DEAE-cellulose-gel H (9:8) in solvent E. [c] R_f values by thin-layer chromatography on gel H in solvent E. [d] R_f values by paper chromatography in solvent F. [e] R_f values by paper chromatography in solvent G. [f] Peptide after first Edman degradation. [g] Peptide after second Edman degradation.

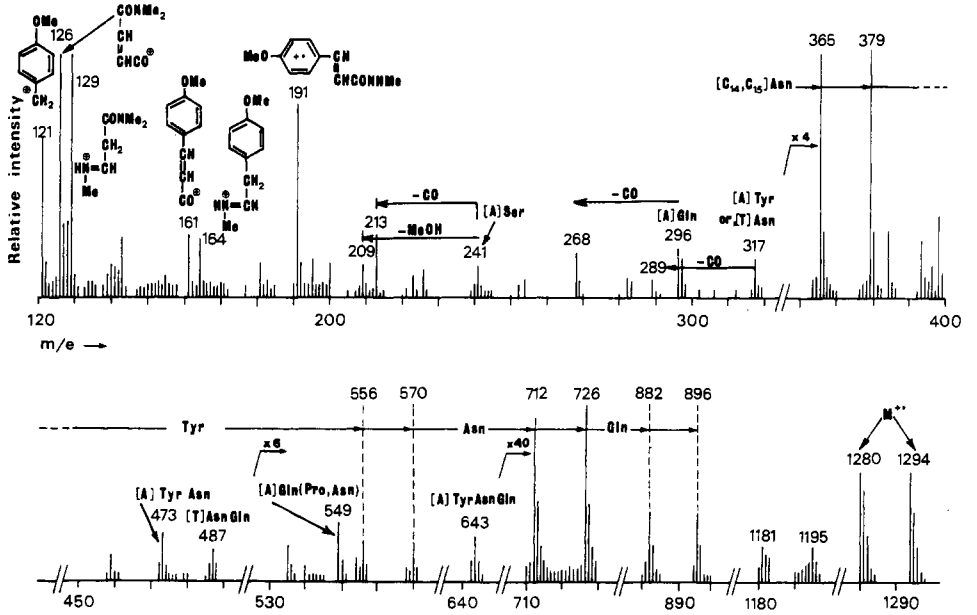


FIGURE 3: Mass spectrum of permethylated iturine A. The symbols [A], [T], and [C_{14},C_{15}] represent the acyl groups originating from the N-C cleavages (see Figure 4) of the methylated Asn, Tyr, and C_{14},C_{15} β-amino acids, respectively.

difference of 51 mass units in the molecular ion peaks indicates the introduction of 17 methyl groups during permethylation and is compatible with the formulas $C_{48}H_{74}N_{12}O_{14}$ and $C_{49}H_{76}N_{12}O_{14}$ for the two unmethylated iturine A homologues together with their amino acid composition. The interpretation of these spectra was simplified by the following observations.

(1) The fragment ions containing C_{14} and C_{15} β-amino acids appear as two homologous peaks with a difference of 14 mass units.

(2) The number of methyl groups introduced during permethylation in a fragment ion is recognized by comparison of the *m/e* values for the permethylated and the perdeuteriomethylated derivatives.

(3) The methylated cyclic peptide chain of iturine A undergoes fragmentation involving N-C cleavages according to the following mechanism (Figure 4). N-C cleavages have been observed previously in permethylated peptides containing His (Morris, 1972), Tyr, Trp, Asn, and Asp (Morris et al., 1971, 1974; Das & Schmid, 1972). Such cleavages are observed in the mass spectra of the permethylated and perdeuteriomethylated iturine A at the Asn and Tyr residues and also

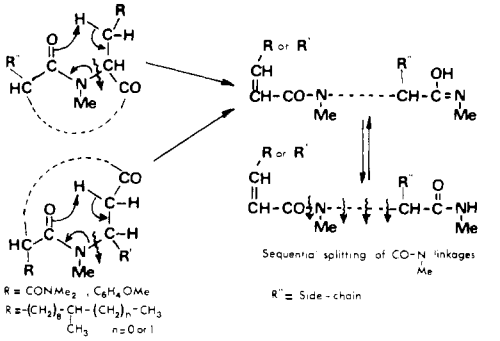
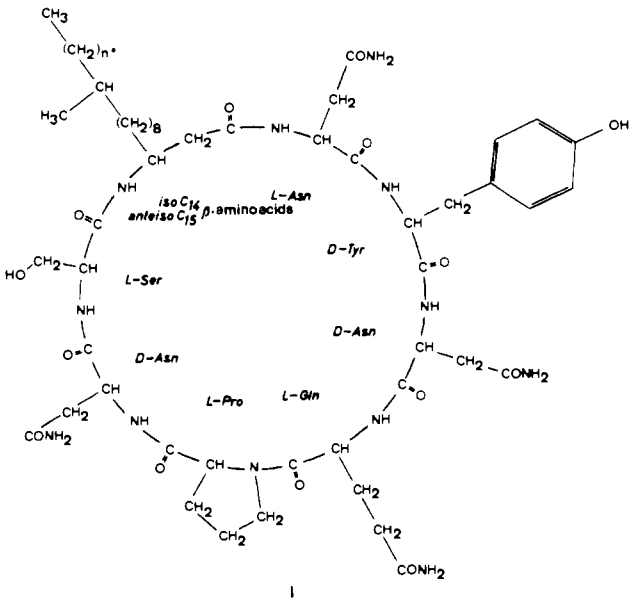


FIGURE 4: Mechanism of N-C cleavages.

at the C_{14},C_{15} β-amino acid level according to a mechanism outlined in Figure 4. The newly observed N-C cleavages of the β-amino acid residues give rise to fragment ions having N-terminal C_{14},C_{15}-acyl groups represented by symbols [C_{14},C_{15}] in Figure 3. The linear peptides resulting from the N-C cleavages of the methylated cyclic peptide exhibit sequence peaks due to splitting of the peptide linkages.

The homologous fragment ions containing the N-terminal C_{14},C_{15}-acyl groups originating from the N-C cleavage of the C_{14},C_{15} β-amino acids as well as the nonhomologous fragment ions containing N-terminal acyl groups originating from the N-C cleavages of Asn or Tyr are indicated in Figure 3.

Various fragment ions thus formed may lose the terminal CO group giving rise to peaks with *m/e* values 28 mass units lower. A methylated Ser residue in a fragment ion loses the elements of methanol, while the permethylated Gln side chain is eliminated from the molecular ions showing the M − [$(CH_3)_2NCOCH{=}CH_2$] peaks at *m/e* 1181 and 1195 for the permethylated iturine A.



I

*n = 0 3-amino 12-methyl tridecanoic acid
n = 1 3-amino 12-methyl tetradecanoic acid

In accordance with the interpretation given in Figure 3, all the peaks are appropriately shifted in the mass spectrum of the perdeuteriomethylated iturine A and these results obtained by mass spectrometry confirm the sequence determined by chemical methods. Therefore, iturine A has the structure I.

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