Structure Determination of a New Cyclodepsipeptide Antibiotic from Fusaria Fungi[†,‡]

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The structure of a cyclodepsipeptide isolated from fusaria fungi has been determined using mass spectrometric, chemical, and chromatographic methods. The antifungal, antibiotic peptide swells *Penicillium digitatum* cells to 10 times their normal diameter and inhibits their germination. The compound has M_r 887 and contains the sequence *d-allo*-Thr-*l*-Ala-*d*-Ala-*l*-Gln-*d*-Tyr-*l*-Leu. 3-Hydroxy-4-methyltetradecanoic acid is linked via an amide bond to threonine and an ester bond to leucine. Fast atom bombardment, field desorption, direct chemical ionization, and electron ionization mass spectrometry and chiral-phase gas chromatography were all employed in the solution of the structure.

Fusaria are plant-infecting fungi that produce a variety of toxins and antibiotics with diverse molecular structures. One important class of biologically active metabolites from fusaria are cyclodepsipeptides. These compounds contain both amide and ester bonds and have been shown to be elaborated by numerous bacteria and fungi (see ref 1 and 2 and references cited therein). We now describe the total structure elucidation of a new cyclodepsipeptide isolated from corn media inoculated with *Fusarium roseum*. This antifungal, antibiotic peptide was originally isolated from *Fusarium roseum* "Acuminatum" NRRL 6227[3] and has since been identified as a metabolite of several other cultivars of *F. roseum* and 18 strains of *F. tricinctum*.[4] When conidia of *P. digitatum* are incubated for 24 h in a culture medium containing the cyclodepsipeptide they swell up to 10 diameters more than conidia incubated in a control medium, and their germination is inhibited.[3,4] This phenomenon is strikingly similar to the effect of funicamycin, an antifungal agent elaborated by *Streptomyces lysosuperificus* that induces *Aspergillus* and *Penicillium* to grow into multinucleate giant cells.[5] The two antifungal agents, however, are not similar in their chemical structure, as tunicamycin does not contain amino acids.[6] Earlier we reported preliminary evidence that the antifungal metabolite produced by *F. roseum* "Acuminatum" is a cyclic hexapeptide linked to an alkyl moiety.[4] The primary structure and enantiomeric composition of this novel cyclodepsipeptide have been elucidated primarily by application of a variety of mass spectrometric, chemical, and chromatographic methods which are discussed in detail below. The general use of these methods for characterization of peptides with unusual structures has been recently reviewed.[7]

Materials and Methods

Nuclear Magnetic Resonance. ^1H and ^{13}C spectra were acquired using Bruker WM 300 and HX 90 instruments for analysis of samples dissolved in CD_3OD.

Mass Spectrometry. Field desorption (FD) and fast atom bombardment (FAB) mass spectra were determined with a MAT 731 instrument, operated at +8 kV accelerating voltage. Benzonitrile-activated carbon emitters and a −6-kV extraction plate voltage were used for FD. For FAB, a neutral beam of xenon atoms was generated with an Ion Tech B11N gun, mounted and operated as previously described.[8] Low-resolution mass spectra were recorded at 1:1000 resolution; accurate mass measurements were made by peak matching at 1:10 000 resolution.

Direct chemical ionization (DCI) mass spectra were obtained as previously described[9] on a Finnigan-MAT 312 (see below) with ammonia as the reagent gas (ion source pressure (6–8) × 10^{-5} torr, temperature 150 °C, electron energy 200 eV).

Gas chromatography–mass spectrometry (GC/MS) instrumentation and experimental conditions are described below in connection with specific procedures, since different systems were used for the various analyses.

High-resolution electron ionization (EI) mass spectra of samples introduced by direct insertion probe were recorded on Iononet photoplates with a CEC 110B instrument, operated at 8-kV accelerating potential, 70-eV electron energy, at 1:12 000 resolution.

Acid Hydrolysis. Samples of the cyclodepsipeptide were dissolved in 0.5 mL of constant boiling HCl (5.7 M, Pierce). The solution was frozen, flushed with nitrogen, degassed, and heated under reduced pressure in a Teflon-sealed glass hydrolysis bulb (Pierce) at 110 °C. A time of 15 min was used for the partial acid hydrolysis experiments while total hydrolysis was effected by heating for between 36 and 72 h.

Amino Acid Analysis. Determination of amino acids was carried out on total acid hydrolyzates obtained by refluxing the cyclodepsipeptide in 6 N HCl for 24 h. Amino acid analysis was performed with a Glenco MM-100 Amino Acid Analyzer using norleucine as an internal

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[‡] Capillary column GC/EI- and CIMS and DCI experiments were carried out in the laboratory of V. N. Reinhold, Harvard School of Public Health.

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standard and ninhydrin for detection.

Derivatization of Blocking Groups. In separate experiments, aliquots of the total acid hydrolysates were extracted 3 times with diethyl ether. The combined organic extracts were taken to dryness under a stream of dry nitrogen and then were either methylated with ethereal diazomethane [10% (vol/vol) methanol for 30 min at 25 °C] or trimethylsilylated by using BSTFA (Regis) and pyridine (5:1) for 30 min at 65 °C. Prior to analysis by GC or GC/MS, the methylated samples were dried under nitrogen and redissolved in a known volume of chloroform, while aliquots of the Me_3Si mixture were analyzed directly.

Hydrogenation Conditions. The combined organic extract was hydrogenated with Adam's catalyst at room temperature for 3 h. This product was methylated with diazomethane and analyzed by GC/MS.

Conversion of Peptide Mixtures to Polyamino Alcohols. The sequence of reactions for the derivatization of a mixture of peptides (obtained in this case by partial acid hydrolysis, see above) to a mixture of *N*-(2,2,2-trifluoro-1,1-dideuterioethyl)-*O*-(trimethylsilyl)polyamino alcohols has been described in detail elsewhere.[10,11] The resulting derivatized mixture was analyzed directly by GC and GC/MS (see below). The high volatility, predictable GC retention behavior, and easily interpretable and predictable mass spectra of these polyamino alcohol derivatives have been thoroughly described elsewhere.[10]

Gas Chromatography and GC/MS. Aliquots of the derivatized extracts of the total peptide hydrolyzates and mixtures of polyamino alcohols obtained as described above were chromatographed on a 6-ft glass column ($^1/_8$-in. i.d.) packed with 3% OV-17 on Gas Chrom. Q (100/120 mesh). The temperature was linearly programmed from 70 to 335 °C at 12 °C/min with a helium flow rate of 30 mL/min.

The GC/MS system used in these particular experiments consisted of a Perkin-Elmer Model 990 gas chromatograph with a flame ionization detector coupled via a single-stage jet separator to a Hitachi–Perkin-Elmer RMU-6L mass spectrometer operated in electron ionization mode at 70 eV. The mass spectrometer was interfaced to an IBM 1800 computer for data acquisition and instrument control. Under computer control the instrument was magnetically scanned every 4.7 s from m/z 28 to 767. After termination of the GC/MS experiment the data were processed, resulting in the production of a set of successive mass spectral scans as well as the associated mass chromatograms. These were filmed automatically, by a 16-mm camera, and also stored on magnetic tape. Retention indices were automatically calculated using internal hydrocarbon standards, and the mass spectra were interpreted with the aid of a computer program.[10]

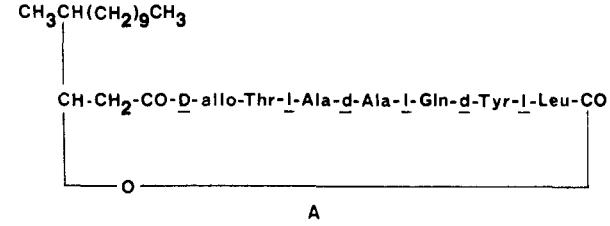
Determination of Absolute Configurations of Amino Acids. Aliquots of the total acid hydrolysates (aqueous phase) were dried in vacuo, converted to the *N*-(pentafluoropropionyl) *N*-propyl esters and chromatographed on a 15 m × 0.25 mm Chirasil-Val fused silica capillary column (Applied Science) as previously described.[12] The column was held at 90 °C for 4 min then linearly programmed at 4 °C/min to 220 °C.

For GC/MS the capillary column was inserted directly into the ion source of the mass spectrometer. The GC/MS computer system consists of a Varian Model 3700 gas chromatograph (equipped with a splitless injector and flame ionization detector) and a Finnigan-MAT 312 double-focusing mass spectrometer fitted with a combined EI/CI/FAB ion source operating in electron ionization mode with an ionization potential of 70 eV. A Finnigan-MAT SS-200 data system controlled the instrument and acquired, processed, and stored the data. For these experiments the mass spectrometer was scanned from m/z 40 to 800 at 1 s per decade.

For chemical ionization ammonia at an ion source pressure of 8×10^{-5} torr was employed (temperature = 150 °C, electron energy 200 eV).

Results and Discussion

Mass spectrometric, chemical, and chromatographic methods contributed to the elucidation of structure A.



A

The nominal molecular weight 887 was determined by low-resolution FD, FAB, and NH_3-DCI MS. A small satellite peak 14 daltons below the parent ions in these mass spectra indicated the presence of a minor amount (<10%) of a lower homologue. The elemental composition $C_{45}H_{73}N_7O_{11}$ was established by high-resolution FAB MS (MH^+ m/z obsd 888.5459, calcd 888.5446). Data from combustion analysis were consistent with these results. Amino acid analysis indicated the presence of 1 mol each of Thr, Glx (Glu or Gln), and Tyr and 2 mol of Ala per mol of peptide, while Leu, Ile, and Val were present at approximately 0.6, 0.3, and 0.1 mol, respectively.

Ile must substitute for Leu in the polypeptide since the MS data indicated that 90% or more of the material had an M_r = 887 and substitution of Ile for any amino acid in the peptide, other than Leu, would have resulted in the appearance of a relatively intense peak offset by the difference in weights of the residues (e.g., Thr = 45, Ile = 57, difference = +12 u). Similarly, Val must also substitute for Leu since only this change would produce the small satellite peak observed 14 u below the parent ion in the FD, FAB, and DCI mass spectra.

The ^1H and ^{13}C NMR data was also in agreement with the cyclodepsipeptide structure A. The ^{13}C NMR proton-noise-decoupled 22.63-MHz spectrum showed 45 resonances. Eight carbonyls had the following shift assignments: 177.6 (Gln-$CONH_2$), 175.9 (Tyr-CO), 175.23 (O-$CONH_2$), 175.04 (Gln-CO), 173.8 (Thr-CO), 173.0 (Ala-CO), 173.0 (Ala-CO), and 172.1 (Ile-CO). The six carbon resonances of the tyrosine moiety appeared at 157.4 (Tyr-C4′), 131.4 (Tyr-C2′,6′), 128.7 (Tyr-C1′), and 116.3 (Tyr-C3′,5′). A DEPT experiment showed the presence of 14 methylene and 21 methine and methyl resonances.

The types and number of available functional groups in the intact molecule were determined by MS and NMR of selected derivatives. For example, the 84-u increment in M_r upon acetylation reflected the incorporation of two acetyl groups. NMR of the acetylated derivative indicated that the reactive sites correspond to secondary (Thr) and phenolic (Tyr) hydroxyl groups. These data, and the lack of reaction with ninhydrin, established that the NH_2-terminus is blocked. Treatment of the peptide with methanol/HCl under conditions which esterify carboxylates

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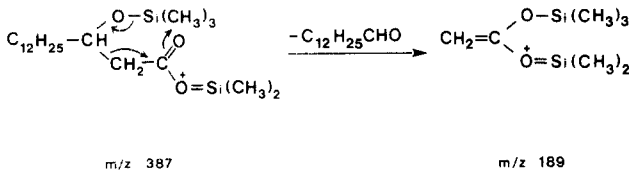
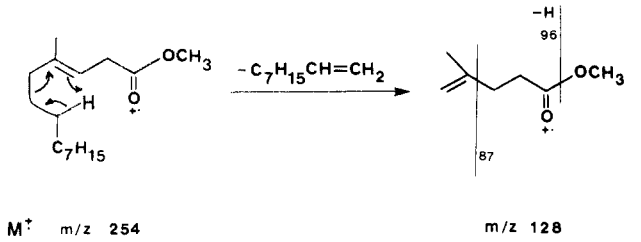
Scheme I. Rearrangement To Form the Ion Observed at m/z 189 in the Mass Spectrum Assigned to the $(Me_3Si)_2$ Derivative of 3-Hydroxy-4-methyltetradecanoic Acid



Scheme II. Rearrangement To Form the Ion Observed at m/z 128 and Its Subsequent Fragmentation in the Mass Spectrum Assigned to 4-Methyl-3-tetradecenoic Acid



failed to shift the apparent M_r of the peptide, indicating the COOH-terminus is also blocked (or cyclic with the NH_2-terminus) and that Glx = Gln. The difference between the composition of the amino acids present ($C_{30}H_{45}N_7O_9$) and the molecular composition indicated the presence of a non amino acid group(s) with the composition $C_{15}H_{28}O_2$.

Hydrolysis of the peptide in 6 N HCl at 110 °C for 36 h released ether-soluble components which were identified by GC/MS following trimethylsilylation or diazomethylation. The structures of the compounds were assigned as 3-hydroxy-4-methyltetradecanoic acid and its dehydration product 4-methyl-3-tetradecenoic acid, on the basis of high- and low-resolution electron ionization mass spectra.

The EI spectrum assigned to the $(Me_3Si)_2$ derivative of 3-hydroxy-4-methyltetradecanoic acid had m/z 73 as its base peak and included peaks corresponding to $(M - CH_3)$ at m/z 387 (21%) and $(M - OMe_3Si)$ at m/z 313 (7%) but lacked a molecular ion. Cleavages adjacent to the Me_3Si ether produced abundant ions at m/z 233 (98%) and 271 (28%), thus ascertaining the β-position of the hydroxyl group. An ion observed at m/z 189 (34%) was probably the result of a rearrangement of the trimethylsilyl group from the β-hydroxyl oxygen to that of the ester carbonyl (Scheme I).

The EI spectrum assigned to the methyl ester of 4-methyl-3-tetradecenoic acid had a molecular ion at m/z 254 (10%) and a fragment due to elimination of methoxy at m/z 223 (14%). Cleavage of the 2,3-bond accompanied by loss of H· resulted in an ion at m/z 180 (19%). Location of the double bond in 3,4-position was suggested by the observation that the base peak in this spectrum (m/z 128) arose by loss of C_9H_{20} with rearrangement of a hydrogen. Further fragmentation then produced ions at m/z 96 (54%) and 87 (42%), as illustrated in Scheme II.

The preference for dehydration to the 3,4-position rather than the conjugated 2,3-position would be due to the methyl group at position 4. This group permitted the formation of a trisubstituted double bond. Appearance of an ion at m/z 114 (28%) gave further evidence for the location of the methyl group.

The identities of these compounds were affirmed by hydrogenation of a portion of the ether-soluble material over platinum dioxide catalyst, followed by diazomethylation and analysis by GC/MS. The mass spectrum

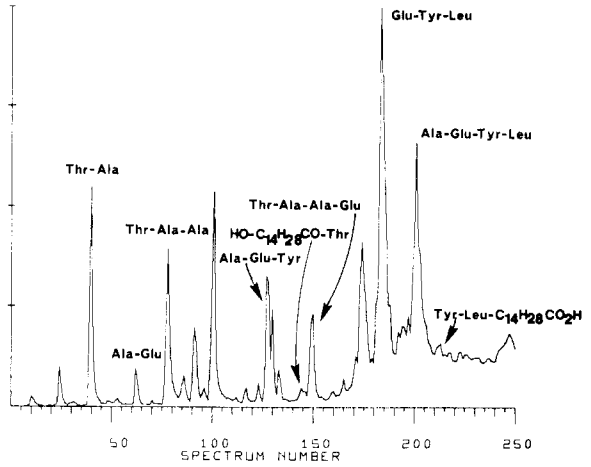
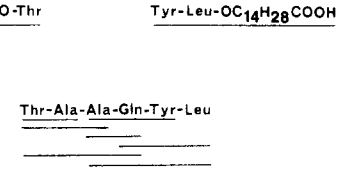


Figure 1. Total ionization profile produced in a GC/MS experiment on a derivatized partial acid hydrolyzate of the cyclodepsipeptide from fusarium. Peptides identified by mass spectrometry are labeled.

Scheme III. Peptide Sequences Identified in the GC/MS Analysis (Figure 1) of the Derivatized Partial Acid Hydrolyzate of the Isolate from Fusaria



of the hydrogenated product was identical with that of authentic methyl 4-methyltetradecanoate. MS and proton NMR data on selected derivatives (see above) demonstrated that the 3-hydroxyl group of the 4-methyltetradecanoic acid moiety is not free in the intact peptide. The observation that this hydroxyl is liberated upon acid hydrolysis suggested that this group is ester linked in the intact molecule, probably to the blocked carboxyl terminus of the peptide. Furthermore, the hydrolytic release of the carboxylate moiety of the fatty acid indicates that this functional group is most likely amide linked to the peptide and thus serves as the blocking group known to be present on the NH_2-terminus.

The sequence of amino acids and the linkage of the hydroxy fatty acid to the peptide were unambiguously established using the GC/MS protein sequencing methodology developed by Biemann and co-workers.[10,11] Partial acid hydrolysis (5.7 N HCl, 110 °C, 15 min) of the cyclic peptide produce a complex mixture of linear peptides which were converted in a number of steps to the corresponding *N*-(2,2,2-trifluoro-1,1-dideuterioethyl)-*O*-(trimethylsilyl)polyamino alcohols. The key step of this procedure is reduction of the amide backbone of the peptide to a repeating ethylenediamine, a modification which drastically decreases their polarity. The resulting complex mixture of polyamino alcohols is injected into the GC/MS where the derivatives are separated and mass spectra of each fraction are obtained in a repetitive scanning mode.

A total ionization plot obtained from the GC/MS experiment on the derivatized cyclic peptide is shown in Figure 1. Interpretation of the data led to identification of seven peptides (Scheme III) two of which, Thr-Ala-Ala-Glx and Ala-Glx-Tyr-Leu, unambiguously established the sequence of the peptide portion of the molecule.

The mass spectrum of the latter peptide is presented in (Figure 2) to illustrate the general features of the po-

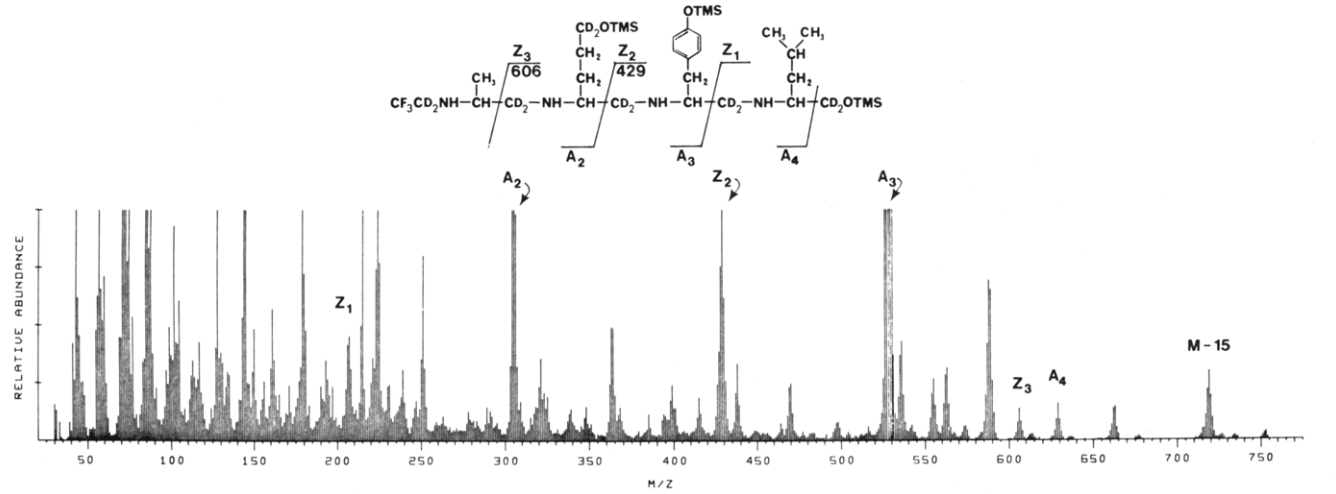


Figure 2. Electron ionization mass spectrum of the component assigned as the Ala-Glu-Tyr-Leu derivative obtained by trifluoroacetylation, reduction with diorane-d_6 and trimethylsilylation of the peptide partial acid hydrolyzate. Gln is converted to Glu during partial acid hydrolysis.

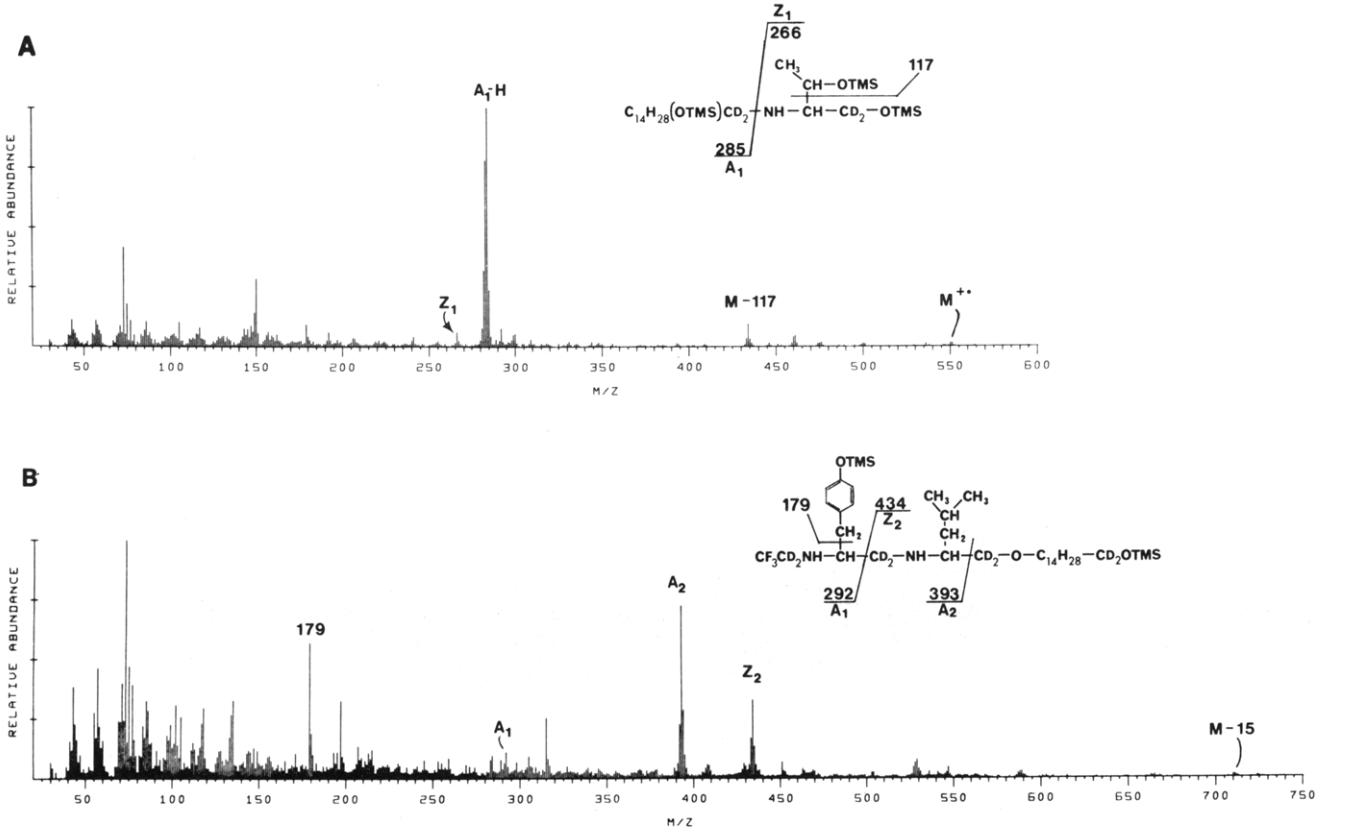


Figure 3. Electron ionization mass spectra of the peptide partial acid hydrolyzate fractions which contained the fatty acid moiety. Shown are the spectra of the derivatives assigned as (A) $C_{14}H_{28}(OH)CO$-Thr and (B) Tyr-Leu-O-$C_{14}H_{28}COOH$.

lyamino alcohol mass spectra. Derivatives of $C_{12}H_{25}CH(OH)CH_2CONH$-Thr and Tyr-Leu-O-$CH(C_{12}H_{25})CH_2COOH$ (Figure 3) were also identified in the GC/MS data, indicating that the peptide is cyclized through the blocking group which is linked via an ester bond to the COOH-terminus and an amide bond to the NH_2-terminus. Survival of the ester during acid hydrolysis and its conversion to an ether rather than an alcohol by reduction in diborane indicates that this bond is sterically hindered.[14] Structural assignments made on the basis of the GC/MS experiments were corroborated by the abundant sequence-related ions observed in FAB mass spectra of the underivatized partial acid hydrolyzates of the peptide. Hydrolysis converts Gln to Glu (difference = +1 u) and apparently causes about 50% ring opening (based on relative peak heights in the FAB data) of the cyclic peptide (difference = 18 u). Thus, the ions observed at m/z 889 and 907 in FAB spectrum (Figure 4) can be assigned as the molecular protonated ions of the cyclic and linear peptides, respectively. Two series of fragment ions dominate the spectrum, which is shown in Figure 4. They correspond formally to the molecular protonated ions of released, linear peptides with either a free carboxyl terminus or with the hydroxy fatty acid moiety attached to the carboxyl terminus (Scheme IV, underlined masses). Formation of these peptides from the cyclic depsipeptide may be relationalized by assuming that the amide bond between Thr and the blocking group and the ester bond between Leu and this group cleave faster under the hydrolysis conditions employed than do the peptide bonds.

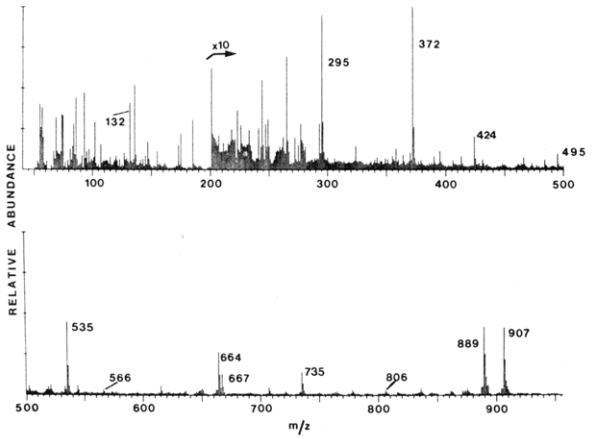
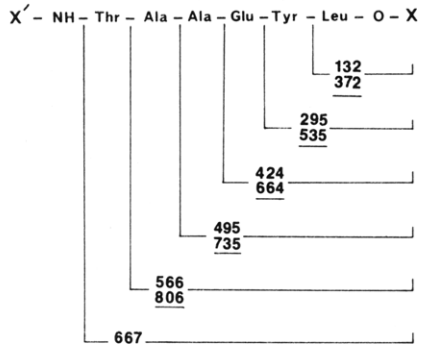


Figure 4. Fast atom bombardment mass spectrum of the underivatized partial acid hydrolyzate of the peptide. Hydrolysis converts Gln to Glu (+1 u) and opens the cyclic structure by addition of water. MN^+ 907, $(M - H_2O)^+$ or MH^+ of unopened peptide 889.

Scheme IV. Assignments of Peaks Observed in the Fast Atom Bombardment Mass Spectrum of the Underivatized Peptide Partial Acid Hydrolyzate from Fusaria[a]



[a] Upper mass values correspond to $(M + H)^+$ for the indicated peptide where X = H and X′ = $C_{14}H_{28}(OH)CO$; underlined masses are for X = $C_{14}H_{28}COOH$ and X′ = H.

Subsequent cleavage of the peptide bonds would then result in the fragments obtained.

The absolute configurations of the amino acids were determined by gas chromatography of the *N*-(pentafluoropropionyl) *N*-propyl ester derivatives on a chiral phase capillary column.[12] Composition and configuration were assigned by comparison of the observed retention times with retention times obtained for the derivatives of *d* and *l* amino acid standards (Figure 5). These results were corroborated by GC/CIMS using ammonia as the reagent gas. These data indicate the presence of one residue each of *d*-Ala, *l*-Ala, *l*-Gln (observed as *l*-Glu), and *d*-Tyr and approximately 0.7 and 0.3 residues respectively of *l*-Leu and *l*-Ile (quantitation based on amino acid analysis data). A small amount of *l*-Val was also observed. Surprisingly, no peak was observed at the expected elution position of *d*- or *l*-Thr. However, an unknown, later eluting peak (11.93 min, +/−0.05 min) had a mass spectrum indistinguishable from that of the derivative of Thr. The unknown was subsequently identified as *d-allo*-Thr by comparison with the retention behavior of standards, including, *d,l-allo*-Thr, *d,l*-Hse, and *d,l*-methyl-Ser.

The remaining question of the sequence of the *d*- and *l*-Ala residues was answered by comparing the relative concentrations of free amino acids present in a partial acid

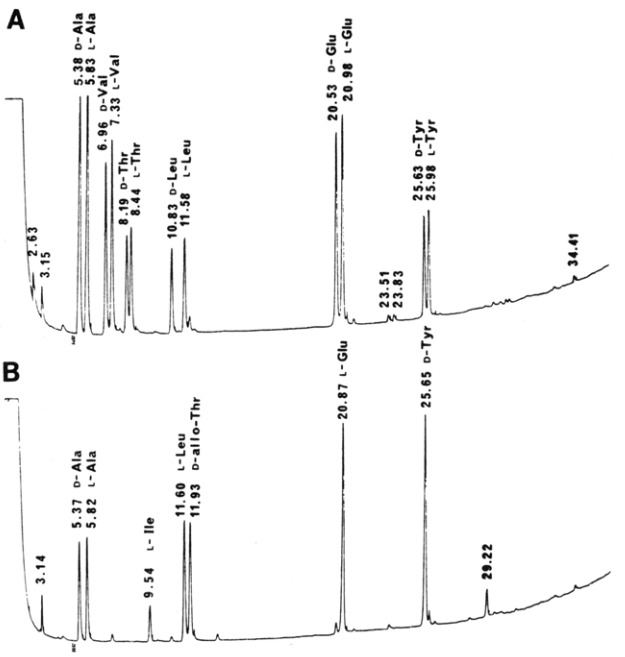


Figure 5. Separation of *N*-(pentafluoropropionyl) *N*-propyl ester derivatives of *d,l*-amino acid enantiomers of a Chirasil-Val fused silica capillary column. For experimental details, see text. (A) Synthetic mixture of six amino acids. (B) Total acid hydrolyzate of the cyclic peptide. Standards of *d,l-allo*-Thr and *d,l*-Ile were analyzed in separate experiments.

hydrolyzate of the peptide before and after treatment of the hydrolyzate with carboxypeptidase A. This enzyme will release *l*-amino acids from the COOH-termini or tri or larger peptides.[13] Hydrolysis conditions identical with those used to produce the mixture shown in Figure 1 were employed. Enzymatic digestion of this hydrolysis mixture only increased the relative peak heights of *l*-Leu and *l*-Glu (and to a lesser extent *l*-Ile and *l*-Val). Since the hydrolyzate was known to contain Thr-Ala-Ala (Scheme III), the failure to observe an increase in the *l*-Ala peak indicates that the probable sequence order is -*l*-Ala-*d*-Ala-. An alternate approach involving saponification of the lactone and treatment of the linear peptide with carboxypeptidase A resulted in the release of *l*-Leu and smaller amounts of *l*-Ile and *l*-Val confirming that the composition of this sequence position is microheterogeneous.

Conclusion

The combined use of mass spectrometric, chemical, and chromatographic techniques constitutes a highly efficient and unambiguous approach for determining the structure of usual peptides. The methods described here enabled the structure of a new, novel cyclodepsipeptide to be elucidated without the necessity for selective opening of the ring to produce a free NH_2-terminus. Furthermore, it was shown that the sequence, structure, and linkage of the blocking group and the absolute configuration of the amino acids could be determined by utilization of GC, FAB, and GC/MS without isolation or purification of degradation fragments.

Registry No. Ala-Glu-Tyr-Leu deriv, 96666-32-9; $C_{14}H_{28}$-(OH)CO-Thr deriv, 96688-74-3; Tyr-Leu-O-$C_{14}H_{28}COOH$ deriv, 96666-33-0; fusaria fungi cyclodepsipeptide, 96666-31-8.

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(14) Lane, C. *Chem. Rev.* **1976**, *76*, 773.