Structure of a Peptidal Antibiotic P168 produced by *Paecilomyces lilacinus* (Thom) Samson

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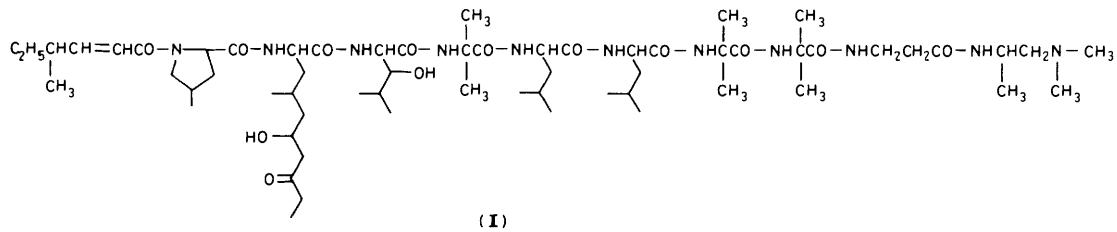
The peptide antibiotic P168 contained a new amino acid, (2*S*,4*S*)-2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid (**6**) and an amine, (*S*)-*N*^{1},*N*^{1}-dimethylpropane-1,2-diamine (**4**) along with other unusual amino acids. The structure of the peptide was determined as (**I**) by in-beam mass spectrometry.

The antibiotic P168 was isolated from a culture filtrate of *Paecilomyces lilacinus* (Thom) Samson and found to possess a wide antimicrobial spectrum against fungi, yeasts, and gram-positive bacteria.[1] The structure of a new amino acid, (2*S*,4*S*)-2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid [2] in the peptide, and the total structure of the peptide [3] have been briefly communicated. In this paper we elucidate the structure of the peptide antibiotic P168 in detail and describe the determination of the stereochemistry of the acid and the amine components.

The antibiotic P168 (**I**), which is basic and isolated as an acetic acid salt,[1] gave an MH^+ ion at *m/z* 1 218 in the field-desorption mass spectrum and revealed 64 carbons including 12 carbonyls in the ^{13}C n.m.r. spectrum (Figure 1). The molecular formula of (**I**) was established as $C_{62}H_{111}N_{11}O_{13}\cdot CH_3CO_2H$ from the above data in conjunction with the elemental analysis.[1] The antibiotic gave no colouration with ninhydrin reagent but its peptide nature was determined from the i.r. spectrum. Further, by acid hydrolysis the antibiotic gave several ninhydrin-positive compounds.



(**I**)
(**II**) CH_2CH_2 instead of CH=CH

The peptide (**I**), as an acetic acid salt, showed two pK_a values in 60% methanol: 5.5 and 8.5. The former could be ascribable to acetic acid as the counter ion of the peptide and the latter was due to a certain tertiary amine; this was deduced by the fact that (**I**) reacted positively with Dragendorff's reagent.

The presence of an (*E*)-α,β-unsaturated amide and an isolated ketone was confirmed from the ^1H n.m.r. (Figure 2) and ^{13}C n.m.r. spectra; δ_C 120.6 (d), 150.3 (d), 165.8 (s), and 211.4 p.p.m. (s). Besides the olefinic protons, the ^1H n.m.r. spectrum indicated signals due to acetyl, *N*,*N*-dimethyl, and nine amide protons.

The main component of the ether-soluble substances of the acid hydrolysate of (**I**) was a γ-lactone, whose structure was determined as 4-hydroxy-4-methylhexanoic acid lactone (**1**) by its mass and n.m.r. spectra, while the dihydro derivative of (**I**), compound (**II**), gave quantitatively (*S*)-4-methylhexanoic acid (**2**) instead of (**1**) after hydrolysis. These data indicate that the acidic moiety of (**I**) is (*S*,*E*)-4-methylhex-2-enoic acid (**3**).

The water-soluble fraction of the acid hydrolysate of (**I**) gave six ninhydrin-positive spots on paper chromatography along with a spot positive with *p*-nitrobenzoyl chloride–pyridine, the reagent for detection of *N*-methyl amino acids.[4] These components were isolated by ion-exchange chromatography [5] and preparative paper chromatography. Among the seven components mentioned above, L-leucine (abbreviated to Leu), α-aminoisobutyric acid (αAib), β-alanine (βAla), *cis*-4-methyl-L-proline [6] (MePro), and *threo*-β-hydroxy-L-leucine [7,8] (HyLeu) were identified by comparison of their physicochemical data with those of authentic samples or references. A quantitative amino acid analysis gave the molar proportions as Leu (2), αAib (3), MePro (1), HyLeu (1), and βAla (1).

The unidentified ninhydrin-positive substance was crystallized as its dihydrochloride, $C_5H_{14}N_2\cdot 2HCl$. Based on the positive colouration with Dragendorff's reagent, it was suggested to be an amine compound containing a tertiary amino group, which was confirmed by *N*,*N*-dimethyl signals in the n.m.r. spectra. The ions at *m/z* 102 (M^+) and 44 in the mass spectrum of the compound shifted to 104 (M^+) and 46 after deuteriation with D_2O. From these data and n.m.r. decoupling results, the structure was determined to be *N*^{1},*N*^{1}-dimethylpropane-1,2-diamine (**4**). The confirmation of structure (**4**) was obtained by synthesis of (**4**) from benzyloxycarbonyl-L-alanine, which was condensed with dimethylamine and the resulting amide was hydrogenolysed. The product, L-alanyl dimethylamide, was reduced with diborane to afford (*S*)-*N*^{1},*N*^{1}-dimethylpropane-1,2-diamine (**4**) which was crystallized as its dihydrochloride. The physicochemical data including the optical rotation of isolated (**4**) were identical with those of synthetic (*S*)-(**4**). Therefore the structure of compound (**4**) was established.

The last component isolated from the hydrolysate, an amino acid $C_{11}H_{19}NO_3$, gave a positive test with *p*-nitrobenzoyl chloride–pyridine reagent, but a negative one with ninhydrin reagent. The mass spectrum gave important fragment ions at *m/z* 168 ($M - CO_2H$), 142 ($M - C_4H_7O$), and 96 (C_6H_9N) along with a molecular ion peak at *m/z* 213. These data suggest that the compound has a substituted piperidine ring. The 400 MHz ^1H n.m.r. spectrum clearly distinguished all the signals, and by decoupling experiments the structure was determined to

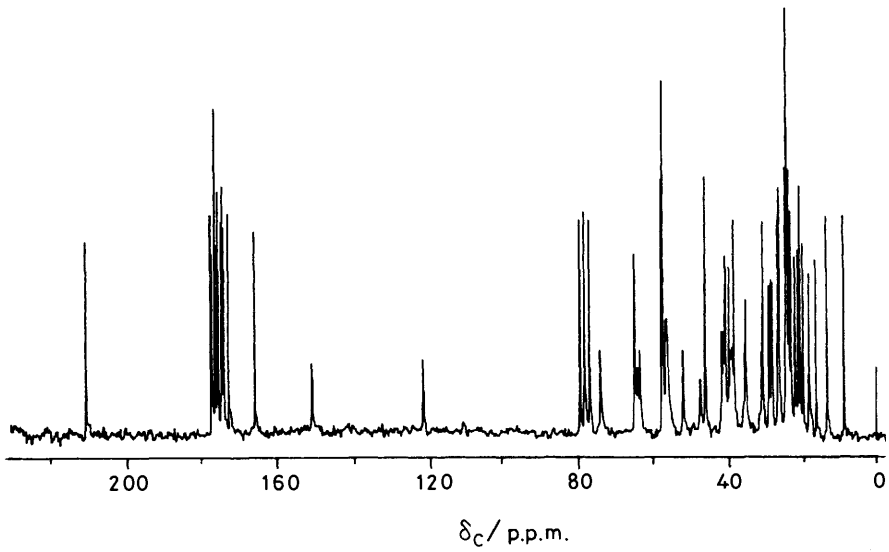


Figure 1. ^{13}C N.m.r. spectrum of P168 (**I**). Concentration: 50 mg in 0.4 ml $CDCl_3$. Data points: 16 K. Spectral width: 6 KHz. Repetition time: 1.5 s. Number of accumulations: 4 000

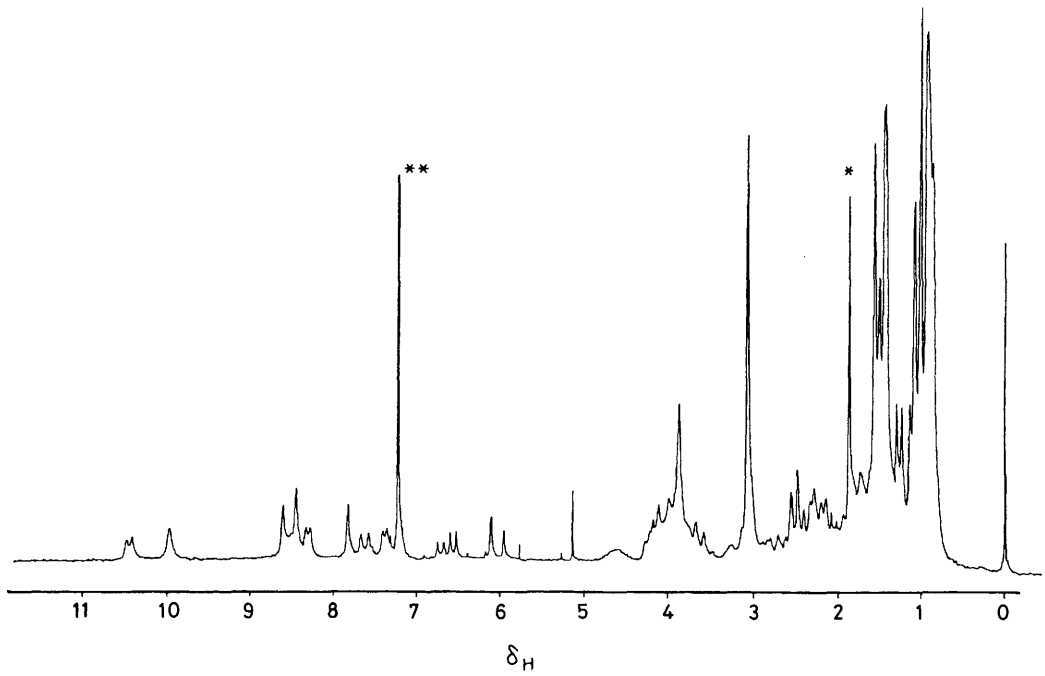


Figure 2. ^{1}H N.m.r. spectrum of P168 (**I**). Concentration: 5 mg in 0.5 ml $CDCl_3$. Data points: 16 K. Spectral width: 1.5 KHz. Repetition time: 10.19 s. Number of accumulations: 80. * Acetate methyl; ** solvent peak

be 4-methyl-6-(2-oxobutyl)piperidine-2-carboxylic acid (**5**) (MOBPC). The ^{1}H and ^{13}C n.m.r. spectra of (**5**) are summarized in the Table. The large coupling constants (13 Hz) of the two double double doublets at δ_H 1.18 and 1.06, 3-H (axial) and 5-H (axial), respectively, indicate that (**5**) takes a chair conformation in water and its three substituents on the piperidine ring should be arranged equatorially. The c.d. spectrum of (**5**) showed a positive Cotton effect at 205 nm, confirming the absolute stereochemistry of C-2 to be *S*,[9,10] and therefore C-4 and C-6 were established as *R* and *R*,* respectively.

Combining these established components one constitutes a peptide with a molecular formula $C_{62}H_{109}N_{11}O_{12}$ (M.W. 1 199) which may have eight amide protons. This is not consistent with the data for (**I**). The difference, H_2O and one amide proton, may be explained if one component thus isolated is an artifact and a dehydration product of a native amino acid, and if the candidate is an imino acid, such as MOBPC or MePro. Some derivatives of (**I**) were then synthesized and precise n.m.r. studies performed. The Scheme and Figure 3 summarize the results.

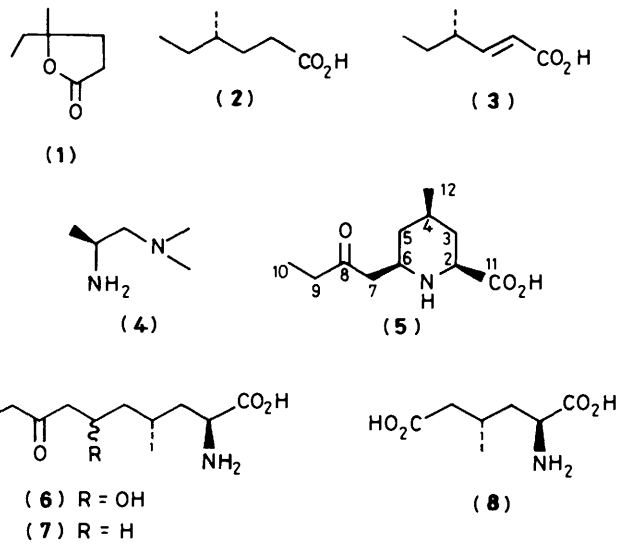
The ^{13}C n.m.r. spectra of (**I**) and (**II**) showed six doublet and one triplet signals between 60 and 80 p.p.m. Among these

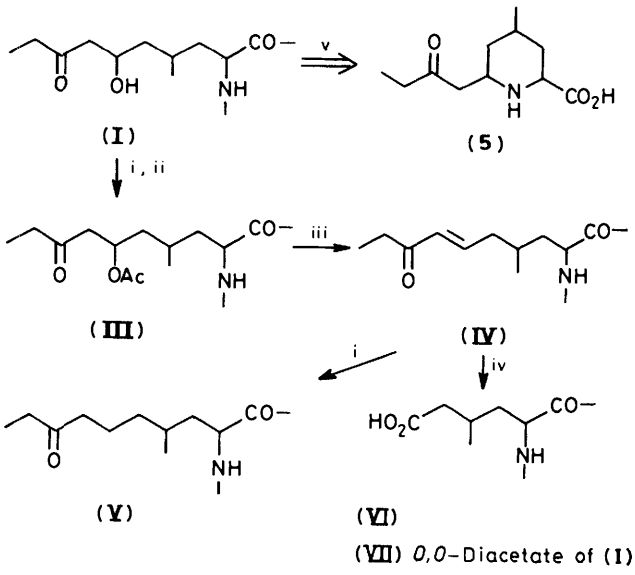
* This amino acid was shown to be identical with the C-6 epimer of trichoponamic acid independently obtained [11] from a hydrolysate of the antibiotic trichoplyn.

Table. ^{13}C and ^{1}H N.m.r. spectra of MOBPC (**5**). Multiplicities in parentheses

	$^{13}C^{a}$	$^{1}H^{b}$	
Carbon	δ (p.p.m.)	δ	*J* (Hz)
2 CH	60.8 (d)	3.58 (dd)	13, 3
3 CH_2	36.6 (t)[c]	2.14 (br d)	13
		1.18 (ddd)	13, 13, 13
4 CH	30.0 (d)	1.78 (m)	
5 CH_2	34.9 (t)[c]	1.80 (br d)	13
		1.06 (ddd)	13, 13, 13
6 CH	52.9 (d)	3.52 (dtd)	13, 6, 3
7 CH_2	44.9 (t)	2.83 (dd)	18, 6
		2.86 (dd)	18, 6
8 CO	214.2 (s)		
9 CH_2	37.0 (t)	2.50 (q)	7
10 CH_3	7.7 (q)	0.94 (t)	7
11 CO_2H	174.9 (s)		
12 CH_3	21.2 (q)	0.90 (d)	6

[a] Obtained at 25 MHz. [b] Obtained at 400 MHz. [c] These assignments may be reversed.





Scheme. Scheme of transformation of (**I**). *Reagents*: i, H_2–PtO_2; ii, Ac_2O–pyridine; iii, benzene–AcOH, heat; iv, $KMnO_4$–$NaIO_4$; v, H^+

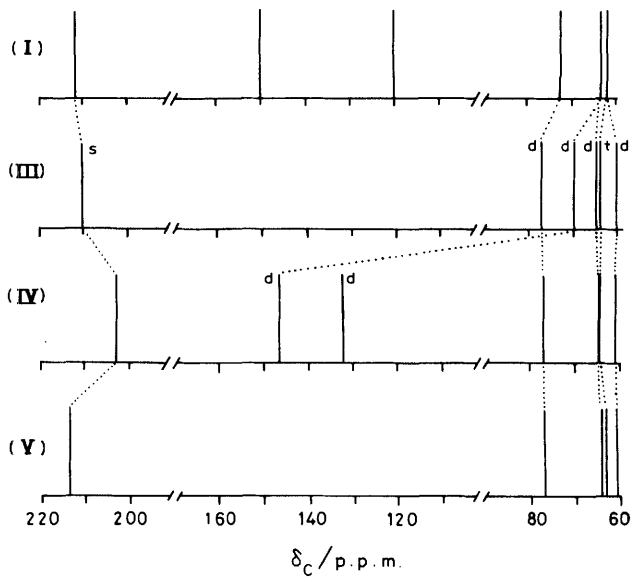


Figure 3. Illustration of ^{13}C n.m.r. spectra of (**I**), (**III**), (**IV**), and (**V**). Signals to lower field than 60 p.p.m. are indicated. Solvent: (**I**), $CDCl_3$; (**III**), (**IV**), and (**V**), CD_3OD

signals two signals (δ_C 72.7 and 63.6 p.p.m.) shifted downfield to δ_C 77.0 and 69.8 p.p.m., respectively, in the *O,O*-diacetate of (**II**), compound (**III**). In the ^{1}H n.m.r. spectrum of (**III**) two acetyl and two acetoxy methine signals were observed. This indicates that two hydroxy functions should be present in (**I**) and (**II**), the one resonating at δ_C 72.7 p.p.m. being that of HyLeu and the other at δ_C 63.6 p.p.m. being unknown. One of the two acetates in (**III**) was shown to be easily eliminated to give (**IV**), an anhydromonoacetyl derivative of (**II**). In the ^{13}C n.m.r. spectrum of (**IV**), the methine carbon at δ_C 69.8 p.p.m. in (**III**) had disappeared and a new pair of olefinic carbons were observed at δ_C 132.6 (d) and 146.4 p.p.m. (d); these carbons were shown to be conjugated with the ketone because the carbonyl carbon resonance in (**III**) (δ_C 210.0 p.p.m.) shifted to 202.9 p.p.m. in (**IV**). In the ^{1}H n.m.r. spectrum of (**IV**), one acetyl signal in (**III**) had disappeared and protons ascribable to a conjugated double bond were observed. This transformation suggests that the amino acid (**5**) having a ketone group is an artifact and that its native precursor contains a hydroxy function α or β to the ketone. To prove this, compound (**IV**) was hydrogenated to give a dihydro derivative (**V**), or was oxidized to afford an acidic derivative (**VI**). In the ^{13}C n.m.r. spectrum of (**V**), the two signals for the olefinic carbons had disappeared and the ketone carbon signal reappeared at lower field (δ_C 213.5 p.p.m.).

From derivatives (**V**) and (**VI**), a new long-chain amino acid (**7**) and an acidic amino acid (**8**), respectively, instead of the amino acid (**5**) should be obtained after acid hydrolysis. Thus, a hydrolysate of (**V**) showed a new ninhydrin-positive spot with a higher R_F value than Leu on t.l.c. The ^{1}H n.m.r. spectrum of the amino acid and the mass spectrum of its *N*-acetyl methyl ester coincided with the proposed structure for compound (**7**). Furthermore, (**7**) showed a positive Cotton effect, at 203 nm, as did (**5**). Thus, (**7**) has the *S* configuration at C-2 and the stereochemistry of compound (**5**) was reconfirmed. Acid hydrolysis of (**VI**) gave an acidic amino acid, which was determined to be 2-amino-4-methylhexanedioic acid (**8**). Thus,

compound (**5**) was shown to be an artifact from a native linear amino acid (**6**) having a hydroxy ketone system. The hydroxy function was determined to be at the β position relative to the carbonyl from the fact that the acetoxy function was easily eliminated in (**III**) and no reaction occurred on periodic acid treatment of (**I**), and the fact that the chemical shift of the hydroxy methine signal (δ_C 63.6 p.p.m.) coincided with the calculated value[12] for 3-oxo-5-hydroxy-7-methyldecane rather than for 3-oxo-4-hydroxy-7-methyl- or 3-oxo-6-hydroxy-7-methyl-decane. Thus, the structure of (**6**), the last component of (**I**), was determined to be (2*S*,4*S*)-2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid (AHMOD), considering the absolute configuration of (**5**). The amino acid (**5**) should be formed from (**6**) through dehydration followed by Michael-type addition during acid hydrolysis.

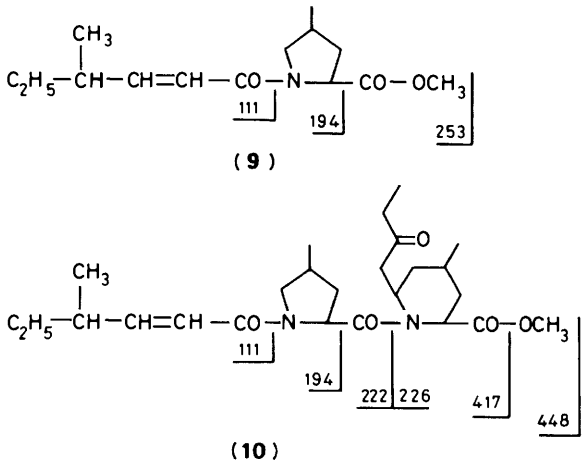
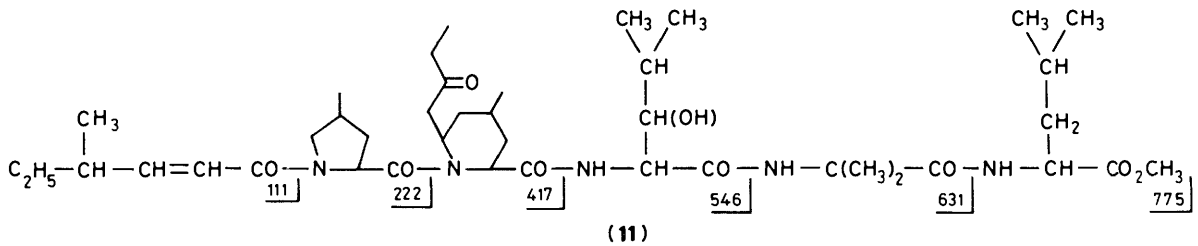


Figure 4. Fragmentations in the mass spectra of fragments A (**9**) and B (**10**)

Thus, all the components of (**I**) were established as being one acid, nine neutral amino acids, and one amine. From the nature of these compounds, the structure of (**I**) was suggested to be a linear peptide blocked with the acid at the N-terminal and with the amine at the C-terminal. When (**I**) was partially hydrolysed with MeOH–HCl, several C-terminal fragments were obtained. Among them, fragments A (**9**), B (**10**), and C (**11**) were isolated and fully characterized. From the mass and n.m.r. spectra and amino acid analysis, fragment A was determined to be (*S*)-*cis*-4-methyl-*N*-[(*E*)-4-methylhex-2-enoyl]-L-prolyl methyl ester (**9**) (Figure 4). Fragment B gave MePro and MOBPC after acid hydrolysis. The mass spectrum of (**10**) can be interpreted from the structure shown (Figure 4). In the ^{1}H n.m.r. spectrum of fragment B no change was observed after addition of trichloroacetyl isocyanate,[13] indicating that AHMOD (**6**) in (**I**) had already cyclized to MOBPC (**5**) in fragment B. Fragment C (**11**) showed a strong peak at *m/z* 703 (*M* − 72) as well as M^+ at 775. This fragment ion was thought to be caused by the elimination of the side chain of HyLeu with rearrangement (C_4H_8O), and was characteristic of a peptide having a β-substituted amino acid. In a usual peptide containing threonine or serine such a rearranged ion has a much lower intensity than the primary cleavage ions.[14] However, the fragment C rearrangement peaks were very strong and became more prominent as the injection temperature was raised. Also, in the field-desorption mass spectrum of (**I**), the peak at *m/z* 1 146 (*M*H − 72), together with the peak for (*M*H − 18), had relatively high intensities at higher emitter current. The sequence in (**11**) was established from the fragmentation pattern, as shown in Figure 5.

Finally the total structure of (**I**) was determined from the in-beam mass spectrum of its *O*,*O*-diacetyl derivative (**VII**). In



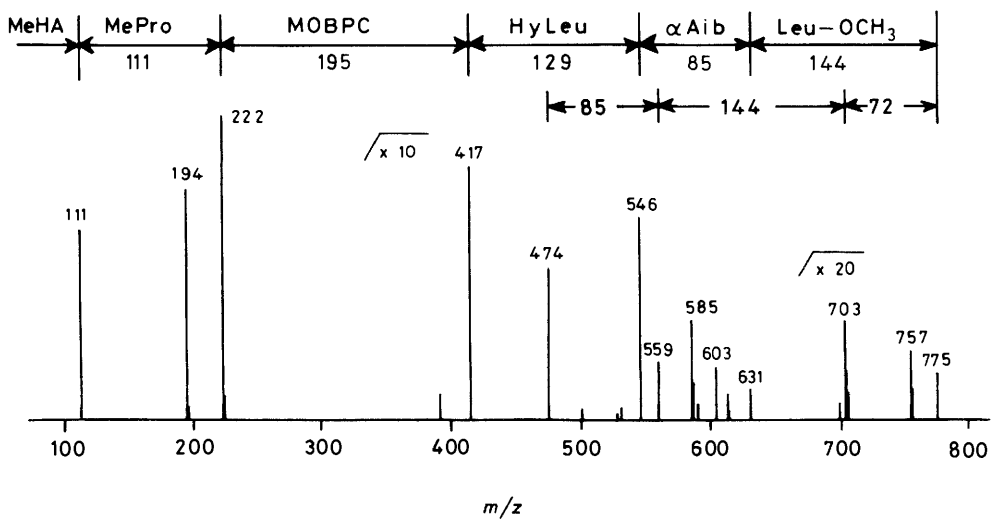


Figure 5. Mass spectrum of fragment C (**11**)

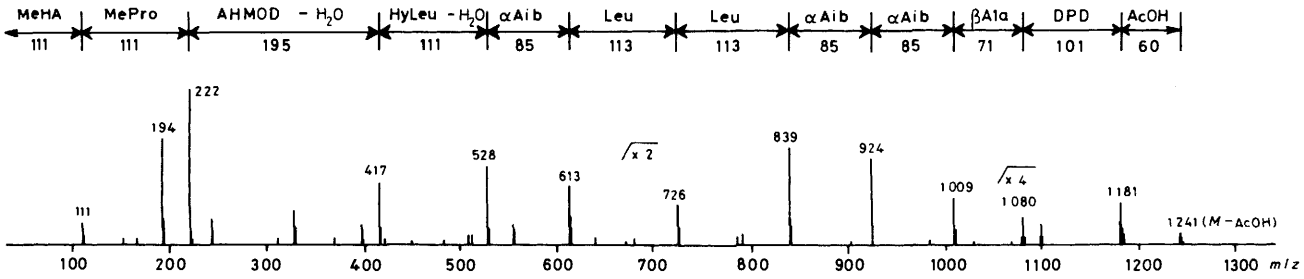


Figure 6. In-beam mass spectrum of the *O,O*-diacetate of (**I**), compound (**VII**). (*S,E*)-4-Methylhex-2-enoic acid (**3**) and (*S*)-N^1,N^1-dimethylpropane-1,2-diamine (**4**) are abbreviated to MeHA and DPD, respectively in the figure. See text for abbreviation of amino acids

view of the sequences of fragments A, B, and C, the mass spectrum of (**VII**) was interpreted as shown in Figure 6. The molecular ion was not obtained, but the highest peak was given at *m/z* 1 241 (*M* − AcOH). As a result the structure of P168 was established as (**I**).

This peptide is unique in that it has many unusual amino acids. In particular, the presence of a new amino acid, (2*S*,4*S*)-2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid, was first established in this peptide. After our preliminary communication [2] the presence of this amino acid in trichopolyn was also confirmed.[11] Leucinostatin A,[15] the structure of which was recently established, is the same compound as P168, and antibiotic I.C.I. No. 13 959 [16] may be also the same as P168. Antibiotic No. 1 907-II [17] is a demethyl analogue of P168.

Experimental

All m.p.s were measured on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were obtained on a JASCO DIP-4 digital polarimeter, and c.d. spectra were recorded on a JASCO J-20 spectrometer. U.v. and i.r. spectra were measured on Cary 14 and JASCO IR-S spectrophotometers, respectively. ^{1}H N.m.r. spectra were recorded with a JEOL JNM FX-100, JEOL JNM MH-100, or JEOL JNM FX-400 spectrometer. ^{13}C N.m.r. spectra were obtained with a JEOL JNM FX-100 spectrometer at 25 MHz using tetramethylsilane or dioxane (δ_C 67.4 p.p.m.) as internal standard. Unit-resolution mass spectra were recorded on a Hitachi RMU-6 mass spectrometer, and high-resolution mass spectra were obtained on a Hitachi H-2 mass spectrometer. In-beam mass spectra and field-desorption mass spectra were measured with a Hitachi IRU-6MG and a JEOL DX-300 mass spectrometer, respectively. Amino acid analysis was performed on a JEOL JLC-200A amino acid analyser.

Isolation of P168 (**I**).—The antibiotic P168 (**I**) was obtained from the culture filtrate of *Paecilomyces lilacinus* (Thom) Samson as already reported,[1] $C_{62}H_{111}N_{11}O_{13}\cdot CH_3CO_2H$, m.p. 111—113 °C; $[\alpha]_D$ −27° (*c* 1 in MeOH); $\lambda_{inf.}$(MeOH) 220 nm(ε23 000); $\nu_{max.}$(Nujol) 3 300 (OH and NH), 1 720 (ketone), and 1 680 cm^{-1} (amide carbonyl); δ_H (100 MHz; $CDCl_3$; see also Figure 2) 1.88 (3 H, s, $CH_3CO_2^-$), 3.10 (6 H, s, 2 × NMe), 6.10 (1 H, d, *J* 16 Hz, CH=C*H*CO), 6.70 (1 H, dd, *J* 16 and 7 Hz, C*H*=CHCO), 7.30 (1 H, d, *J* 5 Hz, NH), 7.52 (1 H, d, *J* 9 Hz, NH), 8.18 (1 H, d, *J* 5 Hz, NH), 8.30 (1 H, s, NH), 8.36 (1 H, br s, NH), 8.50 (1 H, s, NH), 9.76 (1 H, br s, NH), and 10.24 (1 H, d, *J* 6 Hz, NH); δ_C ($CDCl_3$; see also Figure 1), 44.7 (2 × q, NMe), 46.3 (t), 50.8 (t, C-7 of AHMOD), 54.9 (t, C_δ of MePro), 54.9 (d, C_α of Leu), 55.3 (d, C_α of Leu), 56.0 (s, C_α of αAib), 56.2 (s, C_α of αAib), 56.2 (d, C_α of HyLeu), 56.4 (s, C_α of αAib), 62.4 (d, C_α of AHMOD), 62.9 [t, C-3 of (**4**)], 63.6 (d, C_α of MePro), 63.6 (d, C-6 of AHMOD), and 72.8 p.p.m. (d, C-3 of HyLeu); field-desorption m.s., *m/z* 1 218 (*M*H$^+$, 100%), 1 200 (*M*H − 18, 10), and 1 146 (*M*H − 72, 5); pK_a (60% MeOH) 5.5 and 8.5. Colour reactions: positive with Dragendorff's reagent and negative with ninhydrin reagent.

Hydrolysis of (**I**) *and Separation of Each Component.*—P168 (1 g) was hydrolysed with 6M HCl (200 ml) in a sealed tube at 120 °C for 48 h. The hydrolysate was diluted with water and extracted with diethyl ether. The extract was dried (Na_2SO_4), and evaporated to give residual ether-soluble substances (80 mg). The hydrolysate water phase was condensed to dryness under reduced pressure, and the residue was dissolved in water, passed through a Dowex 1 × 2 (OH^-) column, and the column was washed with water. All the eluate was collected, acidified with dilute HCl, and evaporated to dryness. The residue was crystallized from ethanol and gave (S)-N^1,N^1-*dimethylpropane*-1,2-*diamine* (**4**) (60 mg). The ion-exchange column was then eluted with 1M-AcOH and the eluate was condensed under reduced pressure and applied to a Dowex 50 × 8 column pre-equilibrated with pyridine–acetate buffer (0.1M; pH 3.1). The column was developed with a pyridine–acetate buffer system [5] to yield MePro (100 mg), HyLeu (40 mg), αAib (220 mg), Leu (220 mg), and βAla (80 mg). (2S,4R,6R)-4-*Methyl*-6-(2-*oxobutyl*)*piperidine*-2-*carboxylic acid* (*MOBPC*) (**5**) was obtained from the column, mixed with some Leu, and purified with preparative paper chromatography using Toyo filter paper No. 1 and butanol–AcOH–water (4:1:5). The crude MOBPC was crystallized from methanol–acetone and gave pure (**5**) (30 mg).

Identification of Leu, αAib, βAla, MePro, and HyLeu.—These amino acids were identified by comparison of their i.r., n.m.r., and mass spectra with those of authentic samples or published data. Absolute configurations were determined from $[\alpha]_D$ values or c.d. spectra.

L-*Leucine* (*Leu*). $[\alpha]_D$ +15.0° (*c* 2.0 in 6M HCl).

cis-4-*Methyl*-L-*proline* (*MePro*).[6] M.p. 239—242 °C (from 95% EtOH); $[\alpha]_D$ −82° (*c* 1.65 in H_2O; lit.,[6] for D-MePro, + 85.2°); δ_C(D_2O) 16.7 (q, 4-Me), 34.1 (d, C-4), 37.6 (t, C-3), 52.7 (t, C-5), 62.2 (d, C-2), and 175.5 p.p.m. (s, C-1); *m/z* 129 (*M*$^+$) and 84.

β-*Alanine* (*βAla*). δ_C(D_2O) 34.5 (t, C-2 or C-3), 37.4 (t, C-3 or C-2), and 179.2 p.p.m. (s, C-1).

α-*Aminoisobutyric acid* (*αAib*). δ_C(D_2O) 24.5 (2 × q, Me), 59.1 (s, C-2), and 178.7 p.p.m. (s, C-1).

threo-β-*Hydroxy*-L-*leucine*(*Hyleu*).[7,8] M.p. 205—213 °C (from methanol–acetone); θ_{205} +1 200; δ_C(D_2O) 18.1 (q, Me), 19.2 (q, Me), 31.0 (d, C-4), 57.7 (d, C-2), 75.7 (d, C-3), and 174.1 p.p.m. (s, C-1); *m/z* 148 (*M* + 1) and 129.0830 ($C_6H_{11}NO_2$ requires *m/z*, 129.0789). The stereochemistry of the β-hydroxy group was determined as *threo* from the compound's R_F value in paper chromatography [8] and the ^{13}C n.m.r. spectrum compared with those of authentic *erythro*-β-hydroxyleucine,[8] δ_C(D_2O) 19.3 (2 × q, Me), 31.0 (d, C-4), 57.9 (d, C-2), 76.9 (d, C-3), and 172.5 p.p.m. (s, C-1).

4-*Hydroxy*-4-*methylhexanoic Acid Lactone* (**1**).—The ethereal extract of the acid hydrolysate of (**I**) was fractionated into acidic (20 mg) and neutral fractions (50 mg). The neutral compound was pure on t.l.c. (silica gel), $\nu_{max.}$(film) 1 780 cm^{-1} (γ-lactone); δ_H (100 MHz; $CDCl_3$) 0.98 (3 H, t, *J* 7 Hz, 6-H_3), 1.38 (3 H, s, 4-Me), 1.72 (2 H, q, *J* 7 Hz, 5-H_2), 2.0 (2 H, m, 2-H_2), and 2.6 (2 H, m, 3-H_2); *m/z*, 128.0848 (M^+. $C_7H_{12}O_2$ requires *M*, 128.0836), 113.0621 ($C_6H_9O_2$ requires *m/z*, 113.0602), and 99.0451 ($C_5H_7O_2$ requires *m/z*, 99.0445).

(S)-4-*Methylhexanoic Acid* (**2**).—The dihydro derivative of (**I**), compound (**II**) (400 mg), was hydrolysed with 6M HCl as in the case of (**I**) and extracted with diethyl ether. The extract was dried and evaporated to give (*S*)-4-methylhexanoic acid (**2**) (30 mg), $[\alpha]_D$ +10° (*c* 1 in $CHCl_3$; lit.,[18] +6.14°); $\nu_{max.}$(film) 1 710 cm^{-1} (CO_2H); δ_H (100 MHz; $CDCl_3$), 0.90 (3 H, d, *J* 7 Hz, 4-Me), 0.93 (3 H, t, *J* 7 Hz, 6-H_3), 1.1—2.0 (5 H, m, 2- and 3-H_2 and 4-H), 2.35 (2 H, t, *J* 7 Hz, 5-H_2), and 8.7 (1 H, s, CO_2H); δ_C ($CDCl_3$) 11.3 (q, C-6), 18.8 (q, 4-Me), 29.1 (t, C-5), 31.3 (t, C-2 or C-3), 33.0 (t, C-3 or C-2), 34.0 (d, C-4), and 180.8 p.p.m. (s, C-1); m.s. of the methyl ester, *m/z* 144 (M^+), 129, 115, and 113.

Formation of the amide of (**2**). The acid (**2**) (60 mg) was treated with thionyl chloride and then with ammonia to give the amide (27 mg), m.p. 95—96 °C (from hexane) (lit.,[19] 98 °C); $[\alpha]_D$ +9° (*c* 1.5 in $CHCl_3$; lit.,[19] +1.26°).

(S)-N^1,N^1-*Dimethylpropane*-1,2-*diamine* (**4**).—The amine (**4**) was obtained as its dihydrochloride, m.p. 150—151 °C (from EtOH) (Found: C, 33.9; H, 9.1; N, 15.7; Cl, 40.1. $C_5H_{14}N_2$·2HCl requires C, 34.30; H, 9.21; N, 16.00; Cl, 40.50%); $\nu_{max.}$ (Nujol) 1 620, 1 560, 1 540, and 1 220 cm^{-1}; $[\alpha]_D$ −3° (*c* 1.5 in MeOH); δ_H (100 MHz; D_2O) 1.49 (3 H, d, *J* 7 Hz, 3-H_3), 3.03 (6 H, s, 2 × NMe), 3.47 (2 H, d, *J* 7 Hz, 1-H_2), and 4.03 (1 H, quintet, *J* 7 Hz, 2-H); δ_C (D_2O) 17.0 (q, C-3), 43.8 (d, C-2), 44.5 (2 × q, NMe), and 60.3 (t, C-1); *m/z*, 102.1054 (M^+. $C_5H_{14}N_2$ requires *M*, 102.0946), 58.0661 (C_3H_8N requires *m/z*, 58.0657), and 44.0521 (C_2H_6N requires *m/z*, 44.0500).

Formation of the benzamide of (**4**). Treatment of the amine (**4**) (40 mg) with benzoyl chloride (120 mg) in 10% aqueous sodium hydroxide (2 ml) gave the benzamide of (**4**) (48 mg), $[\alpha]_D$ +23° (*c* 2.4 in MeOH); *m/z*, 206.1437 (M^+. $C_{12}H_{18}N_2O$ requires *M*, 206.1418).

Synthesis of (**4**).—*N*-Benzyloxycarbonyl-L-alanine (2 g) was added to a solution of dicyclohexylcarbodi-imide (2 g) in dichloromethane–dioxane (1:1; 20 ml) and the mixture was stirred for 20 min at room temperature. Then dimethylamine was bubbled into the stirred solution for 2 h. The mixture was kept overnight and filtered. The filtrate was extracted with ethyl acetate and the extract was washed successively with 0.25M HCl and 2.5% aqueous sodium carbonate, dried, and concentrated under reduced pressure. The residue was applied to a silica gel column (60 g) and the column was washed with 30% ethyl acetate in hexane and then eluted with 70% ethyl acetate in hexane to give *N*-benzyloxycarbonyl-L-alanine dimethylamide (1.17 g), $[\alpha]_D$ +15.6° (*c* 5.0 in $CHCl_3$); $\nu_{max.}$(film) 3 300 (NH), 1 710 (amide), and 1 640 cm^{-1} (phenyl); δ_H (100 MHz; $CDCl_3$) 1.34 (3 H, s, CMe), 2.99 (3 H, s, NMe), 3.08 (3 H, s, NMe), 4.74 (1 H, m, C_α-H), 5.08 (2 H, s, OCH_2Ph), 6.00 (1 H, d, NH), and 7.2 (5 H, s, Ph); *m/z* 250 (M^+). The amide (760 mg) was catalytically hydrogenolysed over 5% palladium–charcoal (300 mg) in ethanol (20 ml) and acetic acid (5 ml). The reaction mixture was filtered and the filtrate was concentrated under reduced pressure to yield L-alanine dimethylamide acetic acid salt (520 mg) which was used without further purification. The salt (300 mg) was treated in tetrahydrofuran (THF) (5 ml) with a diborane solution in THF by refluxing for 1 h, then 6M HCl (3 ml) was added to the solution which was refluxed for another 30 min. The mixture was concentrated under reduced pressure, the residue was applied to a Dowex 50 × 8 column (H^+; 30 ml), and the column was eluted (gradient) with 150 ml each of 1M HCl and 4M HCl. The eluate having a new ninhydrin-positive spot on t.l.c. (220—260 ml) was collected, concentrated to dryness under reduced pressure, and crystallized from ethanol to afford (*S*)-N^1,N^1-dimethylpropane-1,2-diamine (**4**) (43 mg), whose physicochemical data were identical with those of isolated (**4**). The $[\alpha]_D$ value of the benzamide of synthetic (**4**) was also identical with that of the benzamide from isolated (**4**).

(2S,4R,6R)-4-*Methyl*-6-(2-*oxobutyl*)*piperidine*-2-*carboxylic Acid* (*MOBPC*) (**5**).—The *amino acid* (**5**) was obtained in low yield from the acid hydrolysate [30 mg from 1.0 g of (**I**)], m.p. 195—198 °C (from MeOH–acetone) (Found: C, 61.3; H, 8.7; N, 6.3. $C_{11}H_{19}NO_3$ requires C, 61.94; H, 8.98; N, 6.57%); θ_{205} + 2 500; $\nu_{max.}$(Nujol) 3 000—2 400, 1 720 (ketone), and 1 600 cm^{-1}; n.m.r. (see Table); *m/z*, 213.1321 (M^+, 3%. $C_{11}H_{19}NO_3$ requires *M*, 213.1279), 168.1388 (60; $C_{10}H_{18}NO$ requires *m/z*, 168.1387), 142.0878 (30; $C_7H_{12}NO_2$ requires *m/z*, 142.0867), and 96.0816 (100; $C_6H_{10}N$ requires *m/z*, 96.0812).

Dihydro Derivative of (**I**), *Compound* (**II**).—P168 (**I**) was catalytically hydrogenated over PtO_2 in methanol. The filtrate was concentrated under reduced pressure and the residue was crystallized from benzene–hexane to afford (**II**) quantitatively, m.p. 111—113 °C. In the ^{13}C n.m.r. spectrum a signal at δ_C 165.8 p.p.m. (s) in (**I**) shifted downfield to the usual amide carbonyl region (172—178 p.p.m.) and the signals for a pair of olefinic carbons in (**I**) (δ_C 120.6 and 150.3 p.p.m.) disappeared.

O,O-*Diacetyl Derivative of* (**II**), *Compound* (**III**).—The dihydro derivative (**II**) (400 mg) was acetylated with pyridine–acetic anhydride at 5 °C. The reaction mixture was kept overnight and poured into ice–water containing HCl, then extracted with ethyl acetate. The extract was washed with aqueous sodium hydrogencarbonate, dried, and concentrated under reduced pressure. The residue was purified with a column of LH-20 using methanol–acetic acid (100:1) as eluant to give the diacetyl derivative of (**II**), compound (**III**) (360 mg), δ_H (100 MHz; CD_3OD) 2.08 (3 H, s, CH_3CO), 2.12 (3 H, s, CH_3CO), 5.22 (1 H, t, *J* 6 Hz, C*H*OAc), and 5.30 (1 H, m, C*H*OAc); ^{13}C n.m.r. spectrum (see Figure 3).

Anhydro-O-*monoacetyl Derivative of* (**II**), *Compound* (**IV**).—The *O*,*O*-diacetyl derivative (**III**) (300 mg) was refluxed in benzene–acetic acid (3:1; 20 ml) for 8 h. The mixture was dissolved in ethyl acetate and washed thoroughly with aqueous sodium hydrogencarbonate, dried, and concentrated to give the anhydro-*O*-monoacetyl derivative (**IV**) (270 mg), δ_H (100 MHz; CD_3OD) 2.10 (3 H, s, CH_3CO), 5.22 (1 H, t, *J* 6 Hz, C*H*OAc), 6.12 (1 H, d, *J* 16 Hz, CH=C*H*CO), and 6.82 (1 H, dt, *J* 16 and 7 Hz, C*H*=CHCO); ^{13}C n.m.r. spectrum (see Figure 3).

Deoxy-O-*monoacetyl Derivative of* (**II**), *Compound* (**V**).—The derivative (**IV**) (100 mg) was catalytically hydrogenated over PtO_2 in methanol to give compound (**V**) (90 mg) after purification on an LH-20 column as for (**III**), δ_H (100 MHz; CD_3OD) 2.10 (3 H, s, CH_3CO) and 5.22 (1 H, t, *J* 6 Hz, C*H*OAc); ^{13}C n.m.r. (see Figure 3).

Acidic Derivative of (**II**), *Compound* (**VI**).—The derivative (**IV**) (200 mg) was dissolved in acetone (10 ml) and water (5 ml), solutions of $KMnO_4$ (13 mg) in water (0.5 ml) and $NaIO_4$ (200 mg) in acetone–water (1:1; 2 ml) were added to the solution at 5 °C, and the mixture was kept overnight at that temperature. After filtration, the mixture was concentrated and extracted

with ethyl acetate. The extract was dried and concentrated under reduced pressure. The residue was applied to an LH-20 column using methanol–acetic acid (100:1) and the main fraction was collected, concentrated, and yielded an acidic derivative (**VI**) (150 mg), which was hydrolysed with 6M HCl without being characterized.

(2S,4R)-2-*Amino*-4-*methyl*-8-*oxodecanoic Acid* (**7**).—The derivative (**V**) (60 mg) was hydrolysed in a sealed tube as in the case of (**I**). The hydrolysate was condensed and applied to Toyo filter paper No. 1. The paper was developed with methyl ethyl ketone–pyridine–acetic acid–water (70:15:2:15) and the zone with R_F 0.3—0.4 was cut and eluted with 0.1M NH_4OH. The eluate was concentrated and purified on a short Dowex 50 × 8 (H^+) column. The residue was crystallized from methanol and gave the amino acid (**7**) (5 mg), m.p. 192—194 °C; θ_{203} +290; δ_H (100 MHz; D_2O) 0.90 (3 H, d, *J* 6 Hz, 4-Me), 0.96 (3 H, t, *J* 7 Hz, 10-H_3), 1.0—1.4 (2 H, m), 1.4—1.8 (5 H, m), 2.50 (2 H, q, *J* 7 Hz, 9-H_2), 2.5 (2 H, m, 7-H_2), and 3.84 (1 H, t, *J* 6 Hz, 2-H); m.s. of the acetyl methyl ester, *m/z*, 271 (M^+), 239.1510 ($M - CH_3OH$. $C_{13}H_{21}NO_3$ requires *m/z*, 239.1520), 228.1656 ($M - CH_3CO$. $C_{12}H_{22}NO_3$ requires *m/z*, 228.1598), and 212.1677 ($M - CO_2CH_3$. $C_{12}H_{22}NO_2$ requires *m/z*, 212.1649).

(2S,4S)-2-*Amino*-4-*methylhexanedioic Acid* (**8**).—The acid derivative (**VI**) (200 mg) was hydrolysed in a sealed tube and the hydrolysate was condensed under reduced pressure. The residue was applied to a Dowex 1 × 2 (Ac^-) column and eluted with pyridine–acetate buffer to give an acidic amino acid which was further purified with a short Dowex 50 × 8 (H^+) column and afforded the dioic acid (**8**) as white powder (10 mg), δ_H (100 MHz; D_2O) 1.00 (3 H, d, *J* 6 Hz, 4-Me), 1.8 (2 H, t, *J* 6 Hz, 3-H_2), 2.0 (1 H, m, 4-H), 2.2 (2 H, m, 5-H_2), and 3.8 (1 H, d, *J* 6 Hz, 2-H); δ_C(D_2O) 18.8 (q, 4-Me), 28.7 (d, C-4), 38.6 (t, C-3), 45.3 (t, C-5), 53.8 (d, C-2), 176.1 (s, C-1), and 181.9 p.p.m. (s, C-6); *m/z* 157 ($M - H_2O$), 112, and 69.

Amino Acid Analysis.—P168 (**I**) (2 mg) was hydrolysed with 6M HCl (0.4 ml) at 120 °C for 40 or 90 h in a sealed tube after degassing. The hydrolysate was concentrated under reduced pressure, dissolved in 0.01M HCl and an aliquot was applied to an amino acid analyser. Leu, MePro, HyLeu, αAib, and βAla were used as standards for retention times and colour intensities. Relative molar proportions of these amino acids were as follows; 40 h hydrolysis: MePro (1.0), HyLeu (0.6), αAib (1.8), Leu (1.7), and βAla (0.6); 90 h hydrolysis: MePro (1.0), HyLeu (0.7), αAib (2.5), Leu (1.7), and βAla (0.8). The colour intensity of αAib was about one twentieth of that of Leu.

Partial Hydrolysis of (**I**).—P168 (**I**) (200 mg) was hydrolysed with methanol–conc. HCl (1:1; 4 ml) at 36 °C for 72 h. The mixture was poured into water and extracted with ethyl acetate. The extract was washed with aqueous sodium hydrogencarbonate, dried, and concentrated under reduced pressure to give a mixture of C-terminal fragments (70 mg). The mixture was then fractionated with an LH-20 column [methanol–acetic acid (100:1)] and subjected to preparative t.l.c. [silica gel; chloroform–acetone (10:1) or chloroform–acetone–acetic acid (30:10:2)] which afforded fragment A (6.8 mg), fragment B (3.4 mg), and fragment C (300 μg).

Fragment A (**9**). δ_H (100 MHz; $CDCl_3$) 3.75 (3 H, s, OMe), 4.40 (1 H, dd, *J* 9 and 7 Hz, C_α-H of MePro), 6.05 (1 H, d, *J* 16 Hz, CH=C*H*CO), and 6.84 (1 H, dd, *J* 16 and 8 Hz, C*H*=CHCO); *m/z* 253 (M^+), 194, and 111. On acid hydrolysis, this fragment gave only MePro as an amino acid.

Fragment B (**10**). δ_H (100 MHz; $CDCl_3$) 2.53 (2 H, q, *J* 7 Hz, $COCH_2CH_3$), 3.02 (1 H, dd, *J* 18 and 11 Hz, $NCHCH_AH_BCO$), 3.40 (1 H, br, d, *J* 18 Hz, $NCHCH_AH_BCO$), 4.20 (1 H, dd, *J* 8 and 5 Hz, C_α-H of MePro), 4.6 (1 H, m, 6-H of MOBPC), 4.70 (1 H, t, *J* 8 Hz, 2-H of MOBPC), 6.04 (1 H, d, *J* 16 Hz, CH=C*H*CO), 6.84 (1 H, dd, *J* 16 and 8 Hz, C*H*=CHCO), and 3.75 (3 H, s, OMe); *m/z*, 448.2940 (M^+. $C_{25}H_{40}N_2O_5$ requires *M*, 448.2934), 417.2695 ($C_{24}H_{37}N_2O_4$ requires *m/z*, 417.2749), 226.1444 ($C_{12}H_{20}NO_3$ requires *m/z*, 226.1442), 222.1493 ($C_{13}H_{20}NO_2$ requires *m/z*, 222.1492), 194.1541 ($C_{12}H_{20}NO$ requires *m/z*, 194. 1543), and 111.0810 ($C_7H_{11}O$ requires *m/z*, 111.0809). The acid hydrolysis of compound (**10**) afforded MePro and MOBPC as amino acids.

Fragment C (**11**). Mass spectrum (see Figure 5). On acid hydrolysis compound (**11**) gave MePro, MOBPC, HyLeu, Leu, and αAib as amino acids.

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References

1 A. Isogai, A. Suzuki, S. Higashikawa, S. Kuyama, and S. Tamura, *Agric. Biol. Chem.*, 1981, **45**, 1023.
2 A. Isogai, A. Suzuki, S. Higashikawa, S. Kuyama, and S. Tamura, *Agric. Biol. Chem.*, 1980, **44**, 3029.
3 A. Isogai, A. Suzuki, S. Higashikawa, S. Kuyama, and S. Tamura, *Agric. Biol. Chem.*, 1980, **44**, 3033.
4 Pl. Plattner and V. Nager, *Helv. Chim. Acta*, 1948, **31**, 2203.
5 J. Liebster, K. Kopoldová, and M. Doviášová, *Nature*, 1961, **191**, 1198.
6 J. S. Dalby, G. W. Kenner, and R. C. Sheppard, *J. Chem. Soc.*, 1963, 4387.
7 J. S. Dalby, G. W. Kenner, and R. C. Sheppard, *J. Chem. Soc.*, 1960, 968.
8 S. Futagawa, T. Inui, and T. Shiba, *Bull. Chem. Soc. Jpn.*, 1973, **46**, 3308.
9 L. Fowden, P. M. Scopes, and R. N. Thomas, *J. Chem. Soc. C*, 1971, 833.
10 I. B. Kristensen, P. O. Larsen, and C. E. Olsen, *Tetrahedron*, 1976, **32**, 2799.
11 T. Fujita, Y. Takaishi, A. Okuyama, E. Fujita, and K. Fuji, *J. Chem. Soc., Chem. Commun.*, 1981, 585.
12 J. B. Stothers, 'Carbon-13 NMR Spectroscopy,' Academic Press, New York, 1972, pp. 142 and 145.
13 A. K. Bosen and P. R. Srinivasan, *Tetrahedron*, 1975, **31**, 3025.
14 K. Bieman, in 'Biochemical Application of Mass Spectrometry,' ed. G. R. Waller, Wiley, New York, 1972, p. 420.
15 Y. Mori, M. Tsuboi, M. Suzuki, K. Fukushima, and T. Arai, *J. Chem. Soc., Chem. Commun.*, 1982, **94**.
16 G. W. Kenner and R. C. Sheppard, *Nature*, 1958, **181**, 48.
17 M. Sato, T. Beppu, and K. Arima, *Agric. Biol. Chem.*, 1980, **44**, 3037.
18 P. A. Levenen and R. E. Marker, *J. Biol. Chem.*, 1931, **91**, 405.
19 P. A. Levenen, A. Rothen, and R. E. Marker, *J. Biol. Chem.*, 1936, **115**, 253.

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