117. High Performance Liquid Chromatography (HPLC.) of Natural Products. III [1]. Isolation of New Tripeptides from the Fermentation Broth of *P. Chrysogenum*[1])

by **N. Neuss, R.D. Miller, C.A. Affolder, W. Nakatsukasa, J. Mabe, L.L. Huckstep, N. De La Higuera, A.H. Hunt, J.L. Occolowitz** and **J.H. Gilliam**

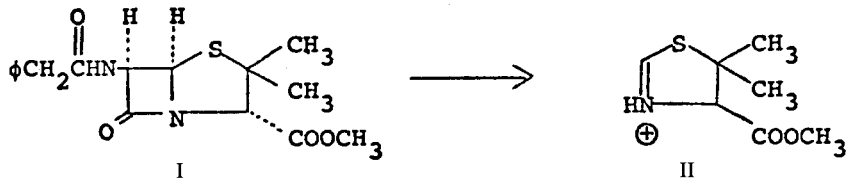
Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana, 46285, U.S.A.

(31.III.80)

Summary

α-Aminoadipoyl-alanyl-valine, α-aminoadipoyl-serinyl-valine and α-aminoadipoyl-serinyl-isodehydrovaline have been isolated from the fermentation broth of *P. chrysogenum*. The configuration of α-aminodipoyl-serinyl-valine has been shown to be L, L, D.

During the isolation of metabolites of *P. chrysogenum* by HPLC. directly from the filtered broth [2], we became interested in one of the least retained fractions eluted from *Microbondapak-NH_2 (Waters)* by acetic acid/methanol/acetonitrile/water 2:4:7.5:86.5 [2]. The complex elution profile that resulted from many UV.-absorbing constituents in the fermentation broth was further analyzed by high resolution mass spectrometry of appropriately derivatized fractions. Fractions were derivatized using phenyl acetyl chloride as an acylating agent and freshly distilled diazomethane for esterification. In order to compare relative amounts of metabolites directly from the peak intensity, we used for mass spectra samples of 5 µl, each containing 40 mg of solids in 1 ml of chloroform or a total of 200 µg per sample. This type of analysis, carried out at different inlet temperatures of the mass spectrometer revealed that the least retained fractions contained varying amounts of substances with prominent peaks at *m/z* 158 and 174 corresponding to empirical formulae of $C_7H_{12}NO_3$ and $C_7H_{12}NO_2S$, respectively. The peak at *m/z* 174 was easily identified as fragment (II) derived from penicillin-G methyl ester (I).



I
Penicillin-G methyl ester

II
m/z 174
$C_7H_{12}NO_2S$

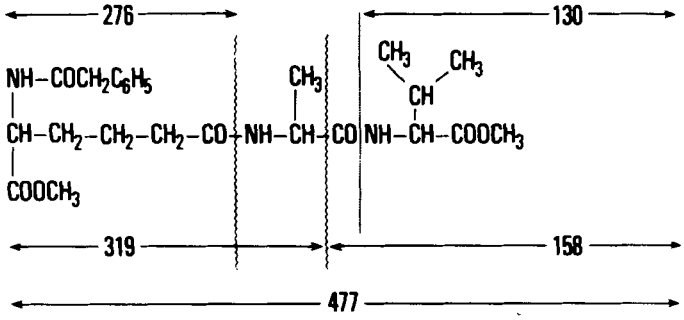
[1]) 1. Mitt.: [1], 2. Mitt.: [2].

0018-019X/80/5/1119-11$01.00/0

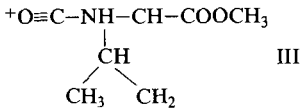
The fragment of *m/z* 158 (III) *Scheme* could not be identified until residues were totally free of *m/z* 174. This was achieved by repeated HPLC. of appropriately selected fractions and led to identification of a new tripeptide, α-aminoadipoyl-alanyl-valine (IV).

NMR. and high resolution mass spectra, as well as amino acid analysis after hydrolysis, corroborate this structure. Although peptides normally are cleaved [3] between CO and NH bonds during mass spectral analyses, in the case of α-aminoadipoyl-alanyl-valine, there seems to be a preferred cleavage leading to the formation of *m/z* 158 rather than *m/z* 130 (see *Scheme*).

Scheme. *Mass Spectrum of α-Aminoadipoyl-alanyl-valine* (high resolution data)

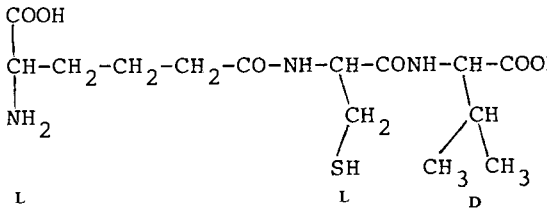


$C_{24}H_{35}N_3O_7$	Calc.	477.2497	$C_{15}H_{18}NO_4$	Calc.	276.127
	Found	477.2473		Found	276.124
$C_{17}H_{23}N_2O_4$	Calc.	319.1666	$C_7H_{12}NO_3$	Calc.	158.082
	Found	319.1657	(III)	Found	158.082

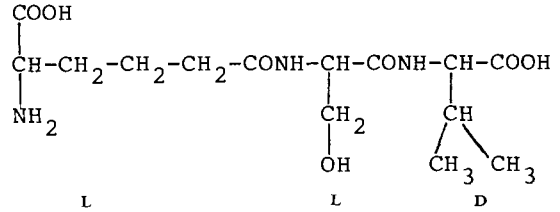


III

The presence of this newly-identified tripeptide in the broth of *P. chrysogenum* is particularly interesting in view of the previous isolation of the only biosynthetically important tripeptide, α-aminoadipoyl-cysteinyl-valine (V). This compound, often called *Arnstein*'s tripeptide, was first reported from the mycelium of *P. chrysogenum* [4]. Unfortunately, it was reportedly contaminated with other peptides containing glutamic acid and glycine [5]. Later it was also isolated from *Cephalosporium* fermentation [6] and shown to have the configuration L, L, D [7–9].



V

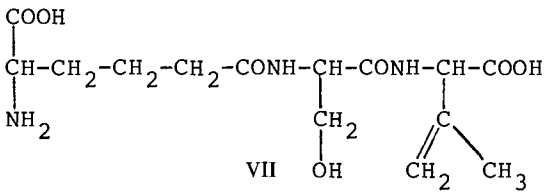


VI

In the process of isolating α-aminoadipoyl-alanyl-valine we noticed the presence of other tripeptides. Eventually it became more convenient to isolate these directly from the broth without subsequent derivatization. In this case we used as a starting material spent beer from the precursed *P. chrysogenum* fermentation from the production of penicillin V. The peptides were first adsorbed on carbon and then eluted with aqueous acetone after washing with water. The appropriate eluates (TLC.) were combined, lyophilized and chromatographed on cellulose to give a mixture of 3–5 peptides (ninhydrin positive). After three consecutive preparative HPLC. runs of this material (two on silica and one on C-18 reverse phase, referred to as A, B and C), the final purification of one peptide from run C was achieved by two analytical HPLC. runs using C-18 reverse phase. The tripeptide was homogenous and shown to be L,L,D-α-aminoadipoyl-serinyl-valine (VI).

The chirality was established by exposing peptide hydrolysate to D- and L-α-aminooxidases [10] and determining residual amino acids. The experiment on serine was inconclusive because this amino acid is not readily oxidized by α-amino-oxidase [11]. Therefore, all three amino acids were isolated after hydrolysis and their CD. spectra recorded and compared with those of authentic optically-pure amino acids.

An early group of fractions from the second preparative HPLC. on silica (B) was combined and rechromatographed on C-18 reverse phase and gave a new tripeptide shown to be α-aminoadipoyl-serinyl-isodehydrovaline (VII).



The NMR. spectrum and mass spectral data were instrumental in establishing the structural features of this new tripeptide (see exper. part). Catalytic hydrogenation of the new tripeptide using PtO_2 in 50% ethanol gave α-amino-adipoyl-serinyl-valine identical in each respect with the authentic material.

We thank the following members of the Lilly Research Laboratories for their help in the course of this work: Dr. *D.E. Dorman,* ^{1}H-NMR. spectra interpretation; Mr. *T.K. Elzey,* recording ^{1}H-NMR. spectra and decoupling experiments; Dr. *G.M. Maciak* and his associates, microhydrogenation and elemental analyses; and Messrs. *R.W. Wetzel,* fermentation; *R.L. King* and his associates, filtration and preliminary purification of the broth; *R.M. Ellis,* amino acid analyses; *S.M. Lawrence,* analytical HPLC. and *Paul D. Vernon,* CD. measurements.

Experimental Part

NMR. spectra were recorded using a *Bruker* WH 360 NMR. spectrometer. *CD. spectra* were recorded in 6N HCl using varying cell paths depending upon the amount of material available using a JASCO Recording Spectropolarimeter J40AS.

In each case the CD. spectrum was identical to that of an authentic optically pure amino acid. *Mass spectra* were recorded using *Varian*-MAT Model 731 mass spectrometer. *Analytical HPLC.* All

chromatograms were obtained using *Waters* M6000A pump, U6K septumless injector (*Waters Assoc.*, Milford, Mass.) with *Schoeffel* Model 770 UV. detector (*Schoeffel* Inst., Westwood, N.J.) and *Fisher* omniscribe recorder (*Fisher Scientific*, Cinncinnati, Ohio). Solvents used were all of the spectral purity type; solvents which were not available commercially were distilled in glass before usage. *Preparative HPLC.* was performed using *Waters Associates Prep* LC/System 500 with Prep Pak 500 Compression Chamber.

Isolation of α-Aminoadipoyl-alanyl-valine (IV). Clear filtrate (60 l) from a 100 l fermentation tank of *P. chrysogenum* was passed over 9 l carbon (DARCO 20×40, buffered at pH 5.5) on a 10-cm column at 150 ml/min flow rate. After 5 h the column was washed with 70 l of distilled water at a maximum flow rate. Following this, the column was eluted with 20 l of 10% aqueous acetone. The first 5 l were discarded

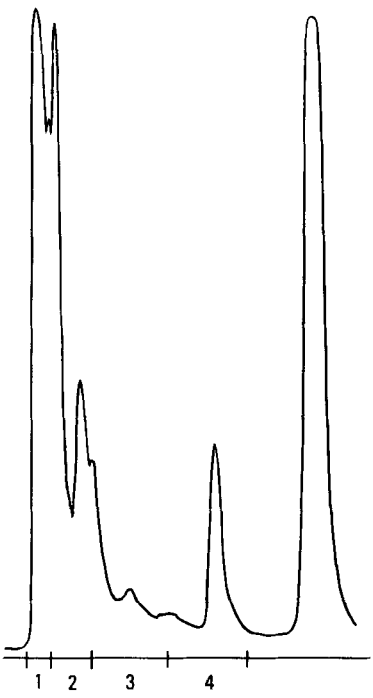


Fig. 1. *Isolation of α-AA-Alanyl-valine I.* Separation of *Prep* 500. Fraction 26,27 (75 µl); UV. range: 254 nm; NL Aufs. Chart speed: 5 mm/min; Flow rate: 2.0 ml/min; Column size: 4×300 mm; Packing: *Microbondapak* C-18; Solvent: H_2O/CH_3OH 1:1; psi: 3100. AA = Aminoadipoyl

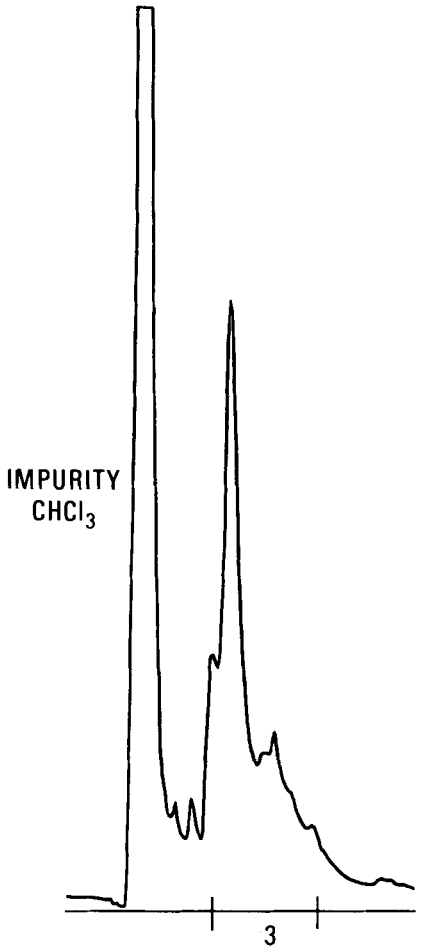


Fig. 2. *Isolation of α-AA-alanyl-valine II.* Analytical reinjection of 25 µl (mCH_3OH); UV. range: 254 nm; 0.08 Aufs. Chart speed: 5 mm/min; Flow rate: 2.0 ml/min; Column size: 4×300 mm; Packing: *Microbondapak* C-18; Solvent: H_2O/CH_3OH 1:1; psi: 4500. AA = Aminoadipoyl

and the remainder was evaporated *in vacuo* to remove the acetone and lyophilized to give 25 g of solids. This material was acylated at 0° with phenylacetyl chloride (2 ml) in 600 ml acetone/water 1:1 in the presence of sodium hydrogencarbonate (25 g). After acidification with conc. hydrochloric acid (pH 2.5), the reaction mixture was extracted with 3×300 ml of ethyl acetate and dried *in vacuo*. The residue was esterified with 7.5 ml of freshly distilled diazomethane and subsequently evaporated *in vacuo* to dryness, yielding 4.25 g of solids. This material was purified using *Waters Prep* 500 on silica catridges with a linear gradient of toluene/ethyl acetate 1:1 to 0:1. Of the 32 fractions collected, those from 22 to 27 exhibited a particularly intense *m/z* 158 peak as compared with the intensity of the *m/z* 174 peak, indicating a relatively high content of peptide IV. Fractions 26 and 27 (after evaporation *in vacuo*) were re-chromatographed on reverse phase *(Microbondapak* C-18 *Waters)* with water/methanol 1:1 as the mobile phase *(Fig. 1)*. Fractions 1, 2, 3 and 4 were collected and subjected to high resolution mass spectrometry. The area designated as 3 showed the highest abundance of *m/z* 158 *(Fig. 2)*. An analogous fraction was then collected from similar chromatographs of fractions 22, 23, 24 and 25 (Prep 500). Ultimately this fraction was sufficiently pure to give an informative NMR. *(Fig. 3)* and a satisfactory mass spectrum *(Scheme)*.

Isolation of L,L,D-α-*Aminoadipoyl-serinyl-valine.* The spent beer from penicillin V production was adjusted to pH 6.9 and filtered to give 190 l of the filtrate. This was adsorbed on 10.5 l of *Pittsburgh carbon* on a (12×40) 10 cm column. The effluent and 40 l of water wash were discarded. Following this, the column was eluted with acetone/water 4:1, collecting 1-l fractions. Fractions 25–40, after evaporation of acetone *in vacuo* and lyophilization, gave 89 g of crude material. This mixture was dissolved in methanol, filtered and adsorbed on 1.5 l of *Avicel* (cellulose); the column was eluted with a mixture of acetonitrile/propanol/water 1:1:0.5 collecting a total of 650 fractions each containing 20 ml.

The composition of fractions was examined by TLC. (silica, BuOH/AcOH/H_2O 2:1:2). Fractions 401–560 were combined. evaporated first *in vacuo,* and then lyophilized, yielding a total of 6.40 g of peptides. This mixture was first purified by preparative HPLC. (A) using *Waters Prep* 500 with two silica cartridges using a linear gradient of 8 l of acetonitrile/water 1:0 to 4:1, followed by an 8 l linear gradient of acetonitrile/water 4:1 to 3:1 and then 2 l of acetonitrile/water 3:1. A total of seventy 250 ml fractions were collected and were combined on the basis of TLC. (ninhydrin positive). This chromatography yielded impure peptides in fractions 44–61 (4.2 g). These peptides were again subjected to *Prep* 500 on silica (B), this time using a linear gradient of 8 l of pure acetonitrile to acetonitrile/water 3:1 and finally 4 l of acetonitrile/water 3:1. Again, fractions of 250 ml were collected, and 27–41 were found to be of the best quality on the basis of TLC. silica, butanol/acetic acid/water 2:1:2. From 27–41 a mixture of 3.5 g

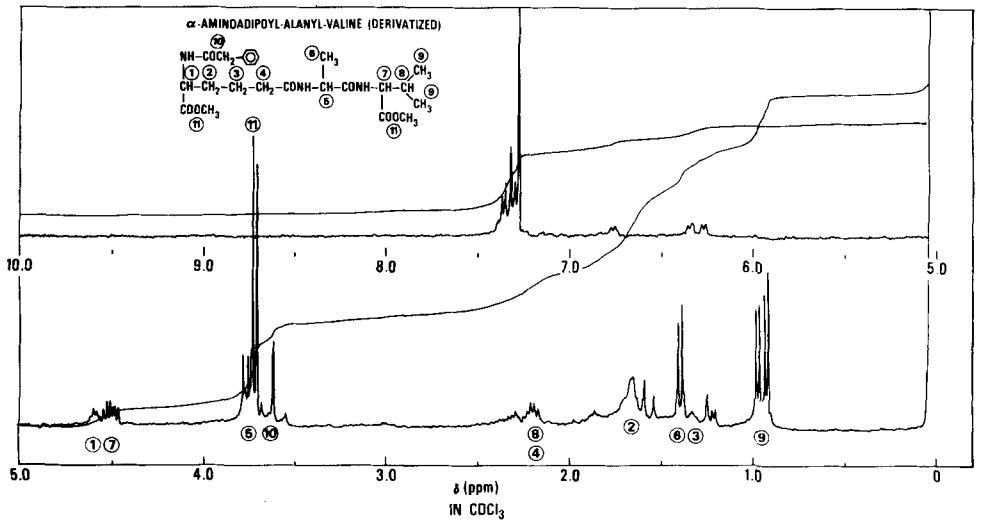


Fig. 3. ^{1}*H-NMR. spectrum of* α-*Aminoadipoyl-alanyl-valine* (derivatized)

of peptides was obtained. This mixture was given a final purification on a C-18 reverse phase *Prep* 500 column (C), using a mixture of 10 l of water, 25 ml of pyridin and 25 ml of acetic acid. A total of 33 fractions, each containing 100 ml, were collected (speed 100 ml/min). On the basis of TLC., fractions 9–32 were combined and concentrated to give 1.4 g of material. The purity of this material was evaluated by an analytical HPLC. run on μ-*Bondapak* (C-18 *Waters*) using 0.4% acetic acid, 0.4% pyridine in water as the mobile phase with refractive index for detection. In a typical run 100 μl of a solution containing 10 mg/ml in water was subjected to HPLC. This was repeated fifteen times to chromatograph 195 mg of material. In such a way, there were obtained 113 mg of peptide from area 2 *(Fig. 4)*. This material still contained traces of pyridine and other poorly defined impurities (NMR.). Therefore, a final purification in a different system was undertaken and is shown in *Figure 5*.

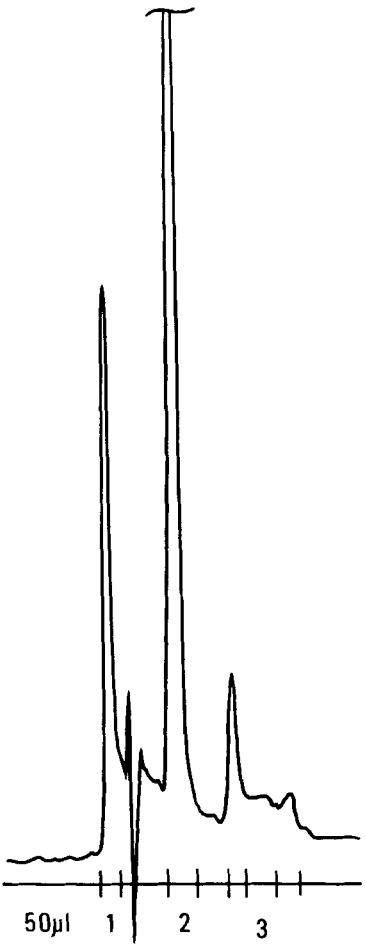


Fig. 4. *Semi-prep. isolation of α-AA-serinyl-valine.* Δri attenuation: 8×; Chart speed: 5 mm/min; Flow rate: 1 ml/min; Column size: 4×250 mm; Packing: *Lichrosorb* RP 18; Solvent: HOAc/Pyridine/H_2O 0.4:0.4:99.2; psi: 1000. AA = Aminoadipoyl

Fig. 5. *Final purification of α-AA-serinyl-valine* (100 μl). UV. range: 254 nm, 0.5 Aufs. Δri attenuation: 8×; Chart speed: 6 mm/min; Flow rate: 2 ml/min; Column size: 4×300 mm; Packing: μ-*Bondapak C-18;* Solvent: HCOOH/CH_3OH/H_2O 0.5:0.5:99; psi: 2000. AA = Aminoadipoyl

The FD. mass spectrum of the purified peptide *(Fig. 6)* as well as its NMR. spectrum *(Fig. 7)* are consistent with structure VI.

In the FD mass spectrum there are fragments containing two hydrogen atoms more than expected. This phenomenon is not unusual as it is apparent from the FD. spectrum of bradykinin [13].

There is more than one possible pathway for the fission leading to *m/z* 118 and *m/z* 205.

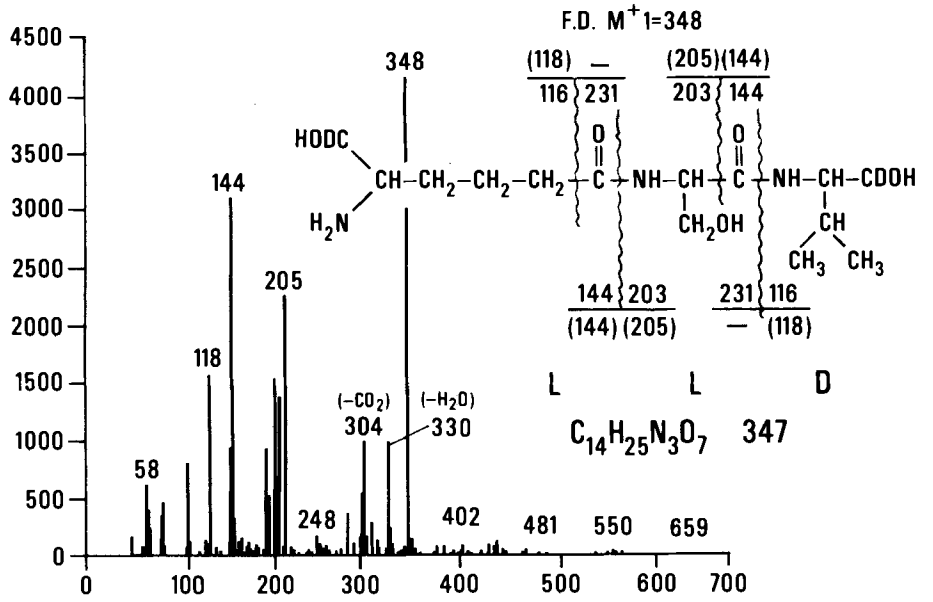


Fig. 6. *Mass Spectrum (FD.) of α-Aminoadipoyl-serinyl-valine*

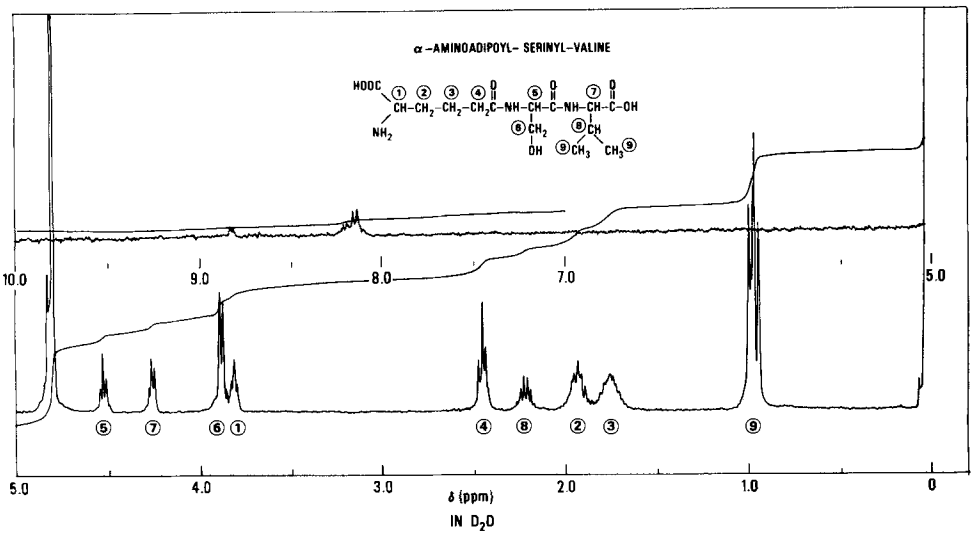


Fig. 7. *^{1}H-NMR. spectrum of α-Aminoadipoyl-serinyl-valine*

The compound was dried for analysis to a constant weight on a block at 120°.

$C_{14}H_{25}N_3O_7 \cdot H_2O$ Calc. C 46.02 H 7.45 N 11.50% Found C 46.43 H 7.10 N 11.66%

Determination of the Absolute Configuration of Amino Acid Residues. The peptide (10 mg) was hydrolyzed with 5 ml of 6N HCl at 110° for 21 h. After removal of hydrochloric acid *in vacuo*, an aliquot of the hydrolysate (0.08 mg) was subjected to amino acid analysis. Another aliquot was treated with L- and D-amino acid oxidase [10], and the deaminated samples were analyzed.

Deamination was performed at 37° for 16 h in a mixture (2.0 l) containing: 1) 1 mg of L-amino acid oxidase in 1.0 ml of 0.4M tris HCl, pH 7.8 and a 1 mg sample of the hydrolysate; 2) 2 mg of D-amino acid oxidase in 1.0 ml of 0.1M sodium pyrophosphate buffer, pH 8.3 and a 1.0 mg sample of hydrolysate.

Table 1. *Determination of the Absolute Configuration of the Amino Acids*

	Amino Acids μmol/mg		
	α-Aminoadipic	Serine	Valine
Hydrolyzed	2.17	2.06	2.45
Deaminated (L-AAO)	0.04	1.54[a])	2.27
Deaminated (D-AAO)	1.91	1.39[a])	0.00
Configuration	L	L	D

[a]) The equivocal result of the action of α-aminooxidase on serine is due to the fact that it is a poor substrate for α-AAO [11].

Fig. 8. *Purification of α-AA-serinyl-isodehydrovaline* (30 μl). Δri attenuation: 8×; Chart speed: 5 mm/min; Flow rate: 2 ml/min; Column size: 4×300 mm; Packing: μ-*Bondapak C-18;* Solvent: $HCOOH/CH_3OH/H_2O$ 0.5:0.5:99; psi: 200. AA = Aminoadipoyl

In addition, all three amino acids were isolated and their CD. spectra recorded.

Isolation of α-Aminoadipoyl-serinyl-isodehydrovaline (VII). Fractions 13–16 from the second preparative HPLC. (B) were combined to give a total of 195 mg. This was subjected to analytical HPLC. using C-18 reverse phase column and pyridine/acetic acid/water 0.5:0.5:99.0. The peptide still contained traces of pyridine in addition to another impurity. Therefore, it was rechromatographed using a C-18 reverse phase column and formic acid/methanol/water 0.5:0.5:99.0 as shown in *Figure 8.* In such a way a total of 35 mg of homogeneous material was obtained.

The comparison of 360-MHz-^{1}H-NMR. spectra in D_2O of the new tripeptide and α-aminoadipoyl-serinyl-valine (VI) *(Table 2)* clearly revealed the presence of α-aminoadipoyl and serine peaks; only the valine resonances were missing. However, there were, in addition, three singlets at $\delta = 5.11$, 4.83 and 1.77. The FD. mass spectrum showed a molecular ion corresponding to $C_{14}H_{23}N_3O_7$, $M+1$ at 346 *(Fig. 9).*

Esterification with diazomethane gave rise to a dimethyl ester, indicating the presence of two carboxyl groups, $M+1$ at 374, $C_{16}H_{27}N_3O_7$ *(Fig. 10).* These data are consistent with structure VII and were confirmed by ^{1}H-NMR. spectra in Me_2SO-d_6 *(Fig. 11).* These indicated that: a) α-CH of α-AAA was not adjacent to the peptide bond (this being the *N*-terminal of the molecule); b) There were two peptide NH doublets; one of these is coupled to the serine α-CH; and the second doublet forms an *AX*-system with a proton that is not coupled any further; c) The only other resonances, which cannot be attributed to α-AAA or serine are singlets at 4.94 (1 H), 4.84 (H), and 1.75 (3 H).

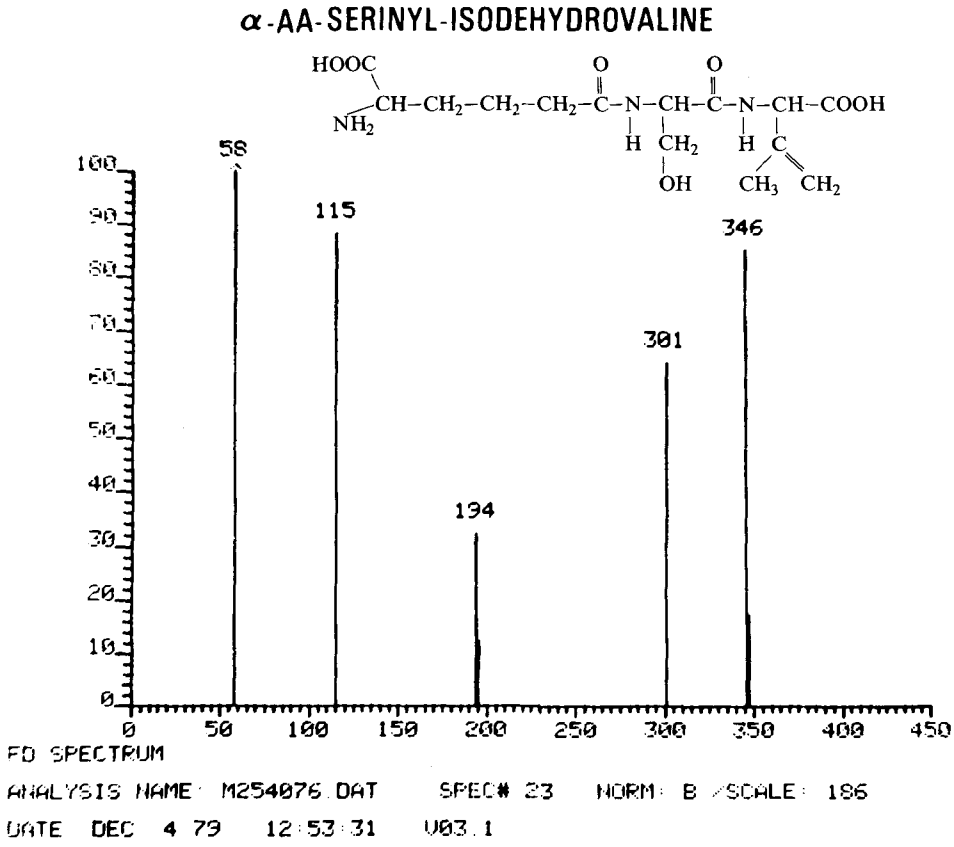


Fig. 9. *Mass spectrum (FD.) of α-Aminoadipoyl-serinyl-isodehydrovaline*

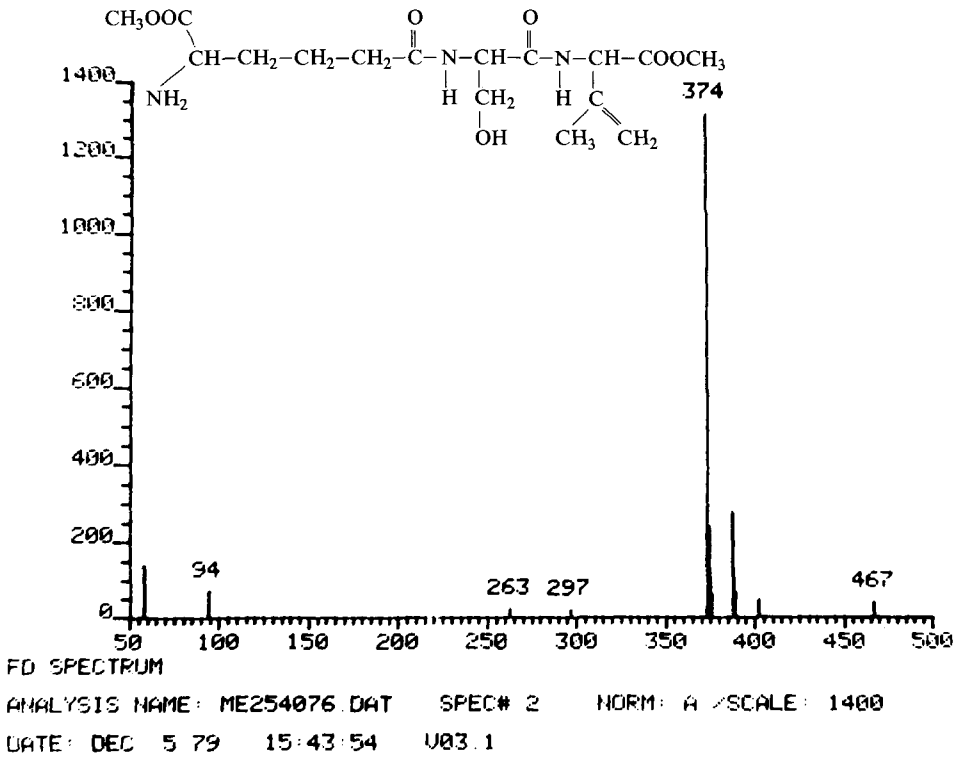


Fig. 10. *Mass spectrum (FD.) of α-Aminoadipoyl-serinyl-isodehydrovaline dimethyl ester*

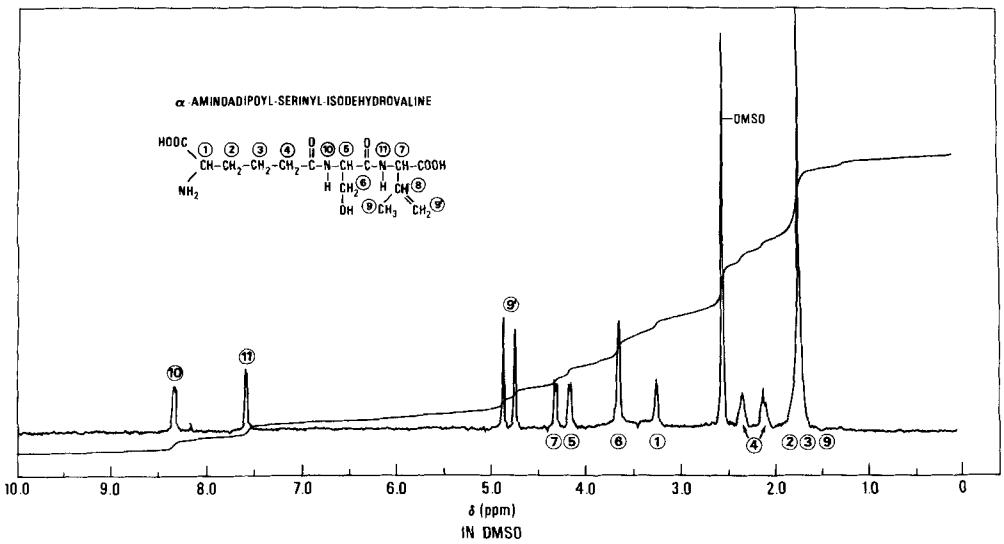
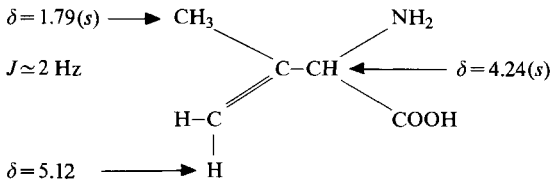


Fig. 11. *^{1}H-NMR. spectrum of α-Aminoadipoyl-serinyl-isodehydrovaline*

Table 2. *Comparison of 360-MHz-^{1}H-NMR. spectra in D_2O* (δ in ppm)

α-AAA-Serinylisodehydrovaline	α-AAA-Serinylvaline	Assignments
5.11 ppm (*s*, 2 H)	–	
4.83 ppm (*s*, 1 H)	–	
4.49 ppm (*t*, 1 H)	4.52 (*t*, 1 H)	Ser α-CH
–	4.25 (*d*, 1 H)	Val α-CH
3.88 ppm (*m*, 2 H)	3.87 (*m*, 2 H)	Ser β-CH_2
3.84 ppm (*t*, 1 H)	3.81 (*t*, 1 H)	AAA α-CH
2.43 ppm (*t*, 2 H)	2.45 (*t*, 2 H)	AAA δ-CH_2
–	2.21 (*m*, 1 H)	Val β-CH
1.93 ppm (*m*, 2 H)	1.93 (*m*, 2 H)	AAA β-CH_2
~1.77 ppm (*m*, 2 H)	1.75 (*m*, 2 H)	AAA γ-CH_2
1.77 ppm (*s*, 3 H)	–	
–	0.98 (two *d*, 0.95 6 H)	Val γ-CH_3's

Isodehydrovaline has been reported to have the following chemical shifts (in D_2O) [14]:



Microhydrogenation of VII. A solution of 4 mg of VII in 50% aqueous ethanol was hydrogenated using 10 mg of PtO_2 for 15 min. The reaction mixture was filtered and lyophilized, yielding 1.8 mg of α-aminoadipoylserinyl-valine. Its NMR. spectrum (360 MHz in D_2O) was indistinguishable from that of an authentic sample.

REFERENCES

[1] *R. D. Miller & N. Neuss,* J. Antibiotics *29,* 902 (1976).
[2] *R. D. Miller & N. Neuss,* ibid. *31,* 1132 (1978).
[3] 'Advanced Methods in Protein Sequence Determination', Ed. Saul B. Needleman, p. 130, Springer Verlag, Berlin 1977.
[4] *H. R. V. Arnstein, M. Artman, D. Morris & E. J. Toms,* Biochem. J. *76,* 353 (1960).
[5] *H. R. V. Arnstein & D. Morris,* Biochem. J. *76,* 357 (1960).
[6] *P. B. Loder & E. P. Abraham,* Biochem. J. *123,* 471 (1971).
[7] *P. A. Fawcett, J. J. Usher, J. A. Huddleston, R. C. Bleaney, J. J. Nishet & E. P. Abraham,* Biochemistry J. *157* (1976).
[8] *J. A. Chan, F. C. Huang & C. J. Sih,* Biochemistry *15,* 177 (1976).
[9] *P. Adriaens, B. Meesschaert, W. Wuyts, H. Vanderhaeghe & E. Eyssen,* Agents and Chemotherapy *8,* 638 (1975).
[10] *D. Wellner & L. L. Lichtenberg,* p. 593 in 'Methods in Enzymology', Vol. *XVIIB,* ed. by H. Taber & C. W. Tabor, Academic Press, New York and London 1971.
[11] *L. L. Lichtenberg & D. Wellner,* Analyt. Biochemistry *26,* 313–319 (1968).
[12] *E. P. Abraham,* J. Antibiot. *30,* 13 (1977).
[13] 'Principles of Field Iomization and Field Desorption Mass Spectrometry' by N. D. Beckey, Pergamon Press, Oxford, page 261, 1977.
[14] *J. E. Baldwin, S. B. Haber, C. Hoskins & L. I. Kruse,* J. org. Chemistry *42,* 1239 (1977).