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CYCLOHEPTAMYCIN, A NEW PEPTIDE ANTIBIOTIC

STRUCTURE DETERMINATION BY MASS SPECTROMETRY*

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Abstract—Cycloheptamycin, an antibiotic from an unidentified Streptomyces species, was examined by mass spectrometry after N-permethylation. The structure was thereby determined as a cyclic derivative of HCO.Val.Thr.-Tyr(Me).Ala.Mealle.β-hydroxyNva.5-methoxyMeTrp.OH, in which a lactone is formed between the C-terminal acid and the threonine OH function. N-methyl*allo*isoleucine and β-hydroxynorvaline were isolated and identified by comparison with authentic compounds, and the configuration of several of the amino acids was determined enzymatically.

DURING our search for new antibiotics from actinomycetes it was found that an unidentified Streptomyces species (MZ 1158), isolated from a soil sample collected near Rize in Turkey, when grown aerobically in deep culture produces a principle which inhibits mycobacteria and certain Gram-positive bacteria. The active principle was extracted from the clarified fermentation broth with ethyl acetate at pH 5 and purified by chromatography of the concentrated extract over silicic acid. It crystallized from ethanol as almost colourless crystals, m.p. 256–258°, $[\alpha]_D^{20} + 37°$ ($c = 1$, $CHCl_3$) and turned out to be a hitherto unrecorded compound for which we propose the name cycloheptamycin.

In addition to cycloheptamycin the extract of the culture fluid contains a number of other compounds three of which have been isolated and identified, viz. N-acetyltyramine, acetyltryptamine, and L-prolyl-L-leucine anhydride. N-Acetyltyramine[1] and L-prolyl-L-leucine[2] have previously been isolated from microorganisms, and acetyltryptamine[3] has been found in the urine of schizophrenic patients. From an acetone extract of the mycelium was isolated a further amount of cycloheptamycin in addition to an antibiotic which could be identified as the known cyclodepsipeptide valinomycin.[4]

Cycloheptamycin is a neutral compound for which the elementary analysis and vaporometric molecular weight determinations suggest the formula $C_{48}H_{68}N_8O_{12}$. In the mass spectrum a molecular ion could not be detected, but the formula was later confirmed by mass spectrometry of a permethylated derivative (*vide infra*). The IR spectrum (KBr) contains bands at 3280 (OH), 1735 (ester or lactone), and a number of amide bonds between 1680 and 1630 cm^{-1}. The UV spectrum (EtOH) (Fig 1) is

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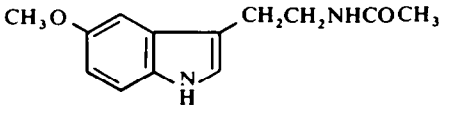
reminiscent of that of the pineal hormone melatonin (**1**) and suggests that the molecule contains a 3-substituted 5-methoxyindole grouping. This view is supported by the mass spectrum which contains a prominent peak at *m/e* 160 characteristic of the ion **2**.[5]



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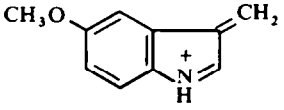

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The NMR spectrum ($CDCl_3$) (Fig 2) of cycloheptamycin is complicated, but contains signals corresponding to two OMe groups (singlets at $\delta = 3{\cdot}75$ and 3·86) and two $-NHCH_3$ groups (broad singlets at $\delta = 2{\cdot}83$ and 3·05). A one proton signal at $\delta = 8{\cdot}75$ suggests the presence of a formyl group.

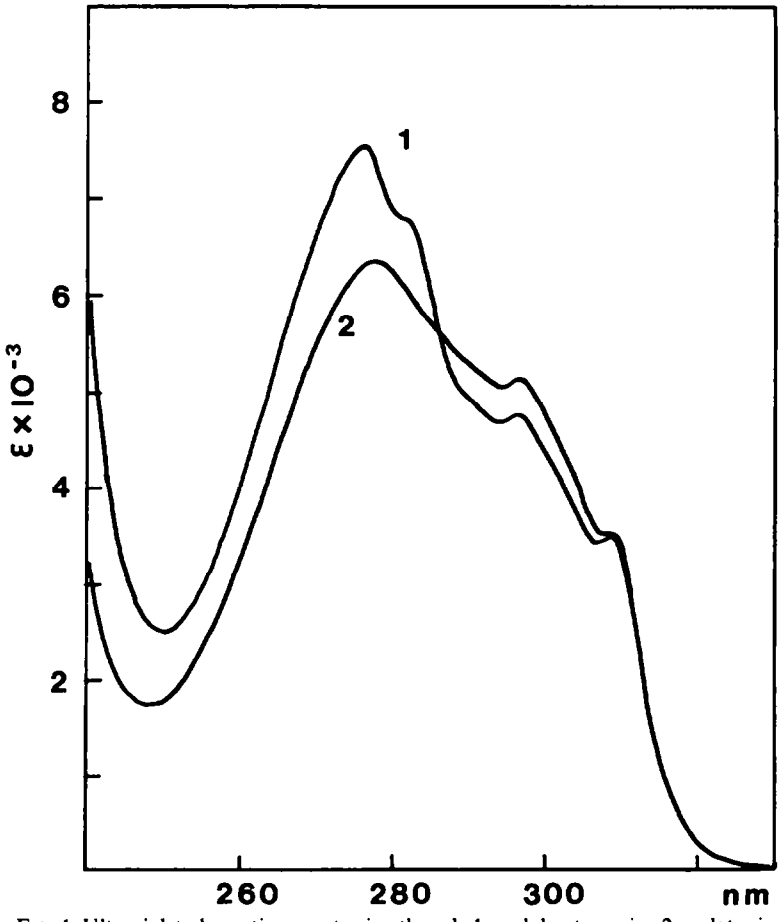


FIG. 1. Ultraviolet absorption spectra in ethanol: 1, cycloheptamycin; 2, melatonin.

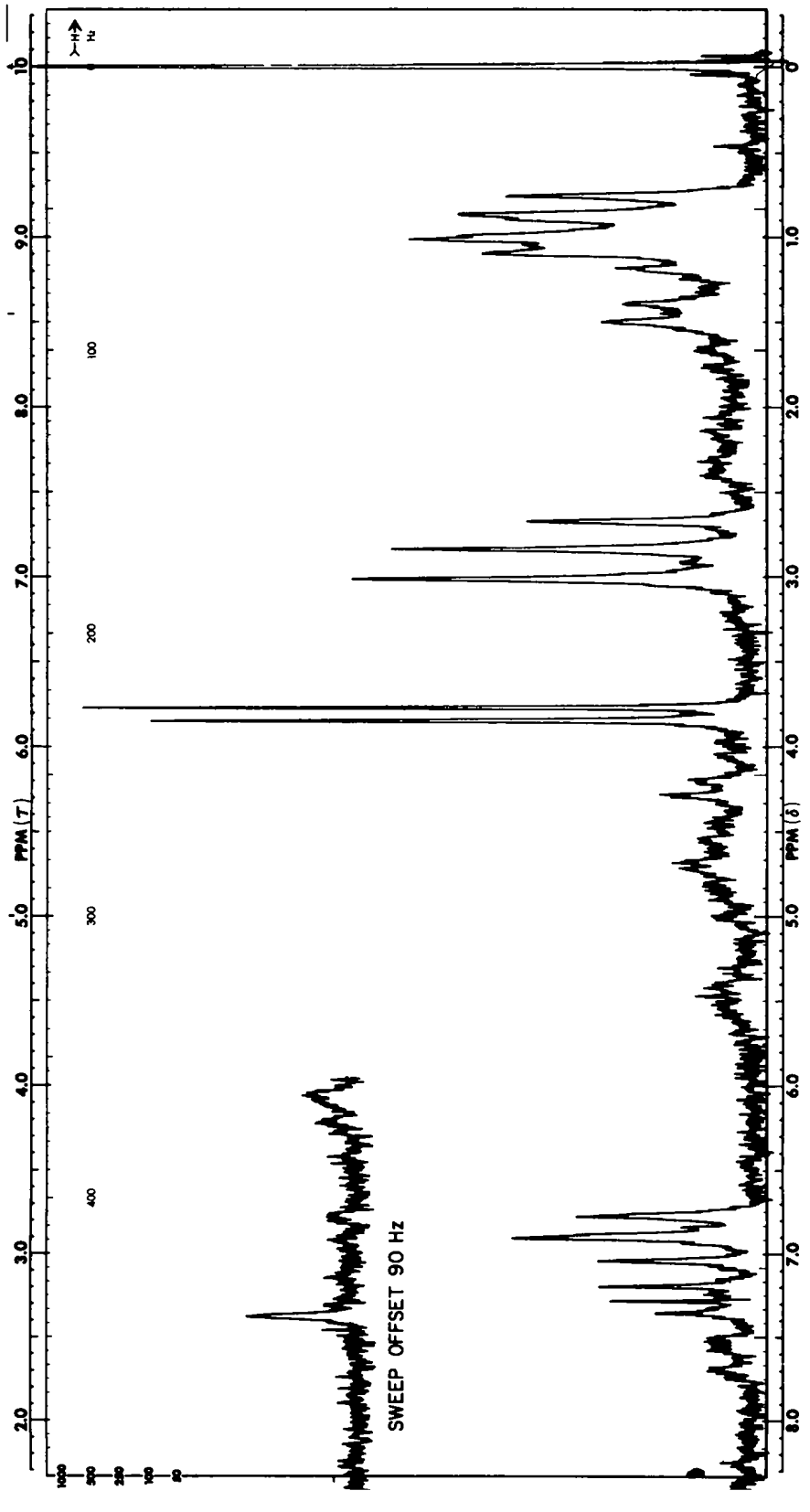
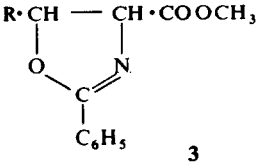


FIG. 2. NMR spectrum of cycloheptamycin in $CDCl_3$.

A Moore Stein analysis of an acid hydrolysate of cycloheptamycin revealed the presence of equivalent amounts of the following amino acids: Valine, threonine,* tyrosine and alanine in addition to an unidentified amino acid positioned between threonine and alanine. Since mass spectrometric investigations (*vide infra*) indicated that this amino acid has the composition $C_5H_{11}NO_3$ the following amino acids were synthesized and compared by TLC with the hydrolysate: β-hydroxyvaline,[6] γ-hydroxyvaline,[7] β-hydroxynorvaline,[8] γ-hydroxynorvaline†[9] and δ-hydroxynorvaline.[10] Of these β-hydroxynorvaline behaved as the unknown amino acid in two different two-dimensional solvent systems as well as in the amino acid analyzer, whereas all of the others readily could be distinguished from this.

β-Hydroxynorvaline contains two asymmetric C atoms, and both of the two possible diastereomeric racemates have been described.[8b, 8c, 11a] In the amino acid analyzer they are readily distinguishable, and the higher melting form turned out to have the same mobility as the isomer present in the hydrolysate. This form has tentatively been assigned the erythro configuration, mainly on the basis of m.p. studies,[8b, 11a] and more recently by IR spectroscopy.[11b] In order to illuminate this point further the oxazolines **3a** and **3b** were prepared by treatment of the methyl esters of the N-benzoyl derivatives of the lower and higher melting racemates respectively, with thionyl chloride.[8b] Comparison of their NMR spectra with those of **3c** and **3d**, prepared analogously from the methyl esters of N-benzoyl-D,L-threonine and N-benzoyl-D,L-*allo*threonine,[12] clearly showed that the oxazoline derived from the higher melting form of D,L-β-hydroxynorvaline is *trans*, and that from the lower melting form *cis* (cf. Fig 3). This demonstrates the correctness of the previous assignment, and the isomer found in cycloheptamycin is consequently an erythro form of β-hydroxynorvaline.



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a: R = Et *cis* form
b: R = Et *trans* form
c: R = Me *cis* form
d: R = Me *trans* form

An amino acid not revealed by the Moore Stein analysis, but detectable in the hydrolysate by TLC was shown by mass-spectrometry to be a N-Me amino acid of the composition $C_7H_{15}NO_2$ (*vide infra*).

In order to identify this the N-Me derivatives of leucine, norleucine, isoleucine and *allo*isoleucine were prepared according to Das *et al.*[13a] and compared by TLC with the hydrolysate using a continuous development technique. In this way N-methylleucine and N-methylnorleucine could be excluded. To distinguish between N-methylisoleucine and N-methyl*allo*isoleucine it became necessary to perform the comparisons with hydrolysates which had been treated with D- and L-amino acid oxidases to remove interfering amino acids. In this way it was shown that N-methyl-*allo*isoleucine was present in the hydrolysate.

* Threonine and *allo*threonine could not be separated in the amino acid analyzer, but by means of TLC it was ascertained that threonine and not *allo*threonine was present in the hydrolysate.

† A sample of this amino acid was kindly provided by Dr. L. Fowden.

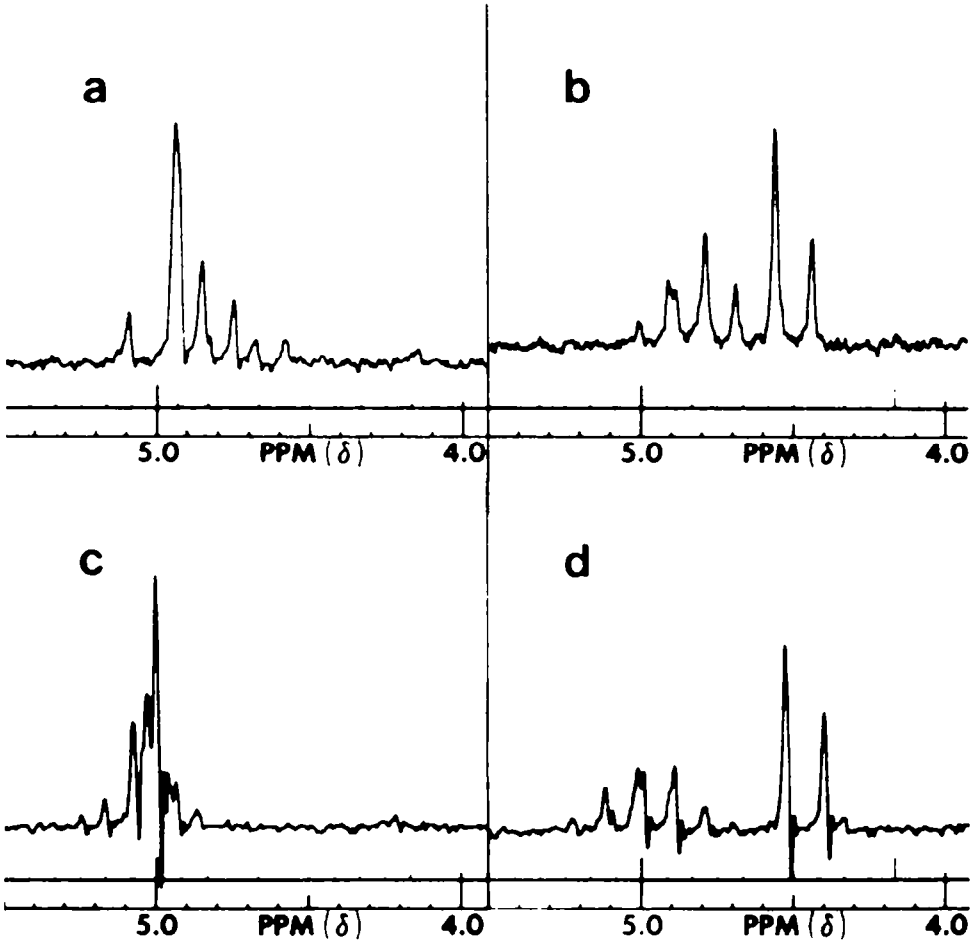


FIG. 3. The C-4 and C-5 proton region of the 60-MHz-spectra ($\delta^{TMS}_{CDCl_3}$) of the oxazolines 3a–d

The question of the configuration of the seven amino acids in cycloheptamycin has been approached enzymically. An acid hydrolysate of the compound was divided in three parts one of which was subjected to the action of hog kidney D-amino acid oxidase and another one to rattlesnake (crotalus terrificus) venom L-amino acid oxidase. The third part was left untreated. TLC and Moore Stein analyses of the three samples (cf. Fig 4) showed that tyrosine and alanine were absent in the sample treated with D-amino acid oxidase, but present in the two other samples. It can therefore be concluded that these two amino acids have the D-configuration. Valine and β-hydroxynorvaline were absent in the sample treated with L-amino acid oxidase, but present in the two other samples. Consequently they belong to the L-series. Threonine was present in all of the samples. Since it is known that the L-isomer of this amino acid is virtually resistant to the influence of rattlesnake L-amino acid oxidase and that the D-isomer is attacked only very slowly by hog kidney D-amino acid oxidase[14] it is not possible to draw any conclusions as to the configuration of the threonine moiety from this experiment. The N-methyl*allo*isoleucine enantiomer

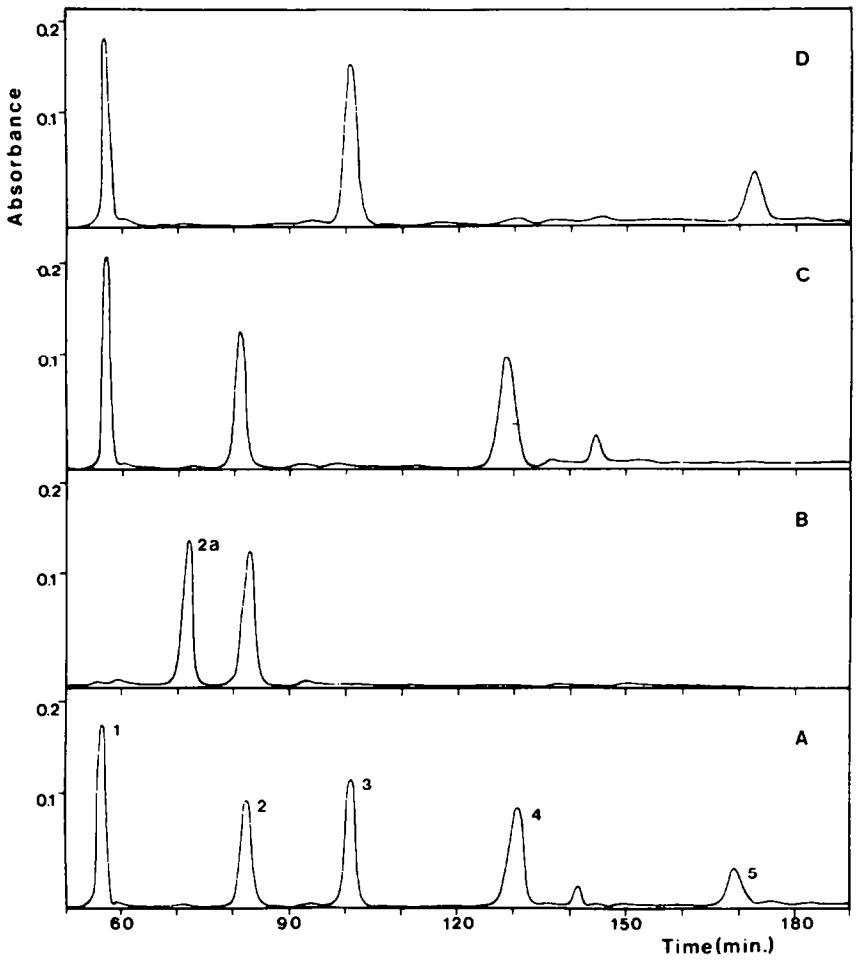


FIG. 4. Moore Stein chromatograms. A, Untreated hydrolysate: Threonine (1), erythro-β-hydroxynorvaline (2), alanine (3), valine (4) and tyrosine (5); B, Threo- (2a) and erythro-β-hydroxynorvaline; C, DAO-treated hydrolysate; D, LAO-treated hydrolysate

was likewise unaffected by both amino acid oxidases as revealed by the fact that the spots on TLC's of all three samples apparently had the same intensity. Therefore, no conclusions as to the configuration of this amino acid can be drawn. An assignment of configuration to the N-methyl-5-methoxytryptophane residue has not been possible since this amino acid is destroyed during the acid hydrolysis.

For determination of the structure of cycloheptamycin, conventional methods were thought to be unsuitable because of limited available amounts of the antibiotic. As an alternative, more sensitive technique, mass spectrometry was considered, but initial attempts were unsuccessful due to insufficient volatility of the compound. This problem could be overcome by N-permethylation—a technique which had been developed to increase the volatility of peptide derivatives specifically for analysis by mass spectrometry,[13] and which has recently been applied to the structure determination of the peptide antibiotics stendomycin[15] and esperin.[16]

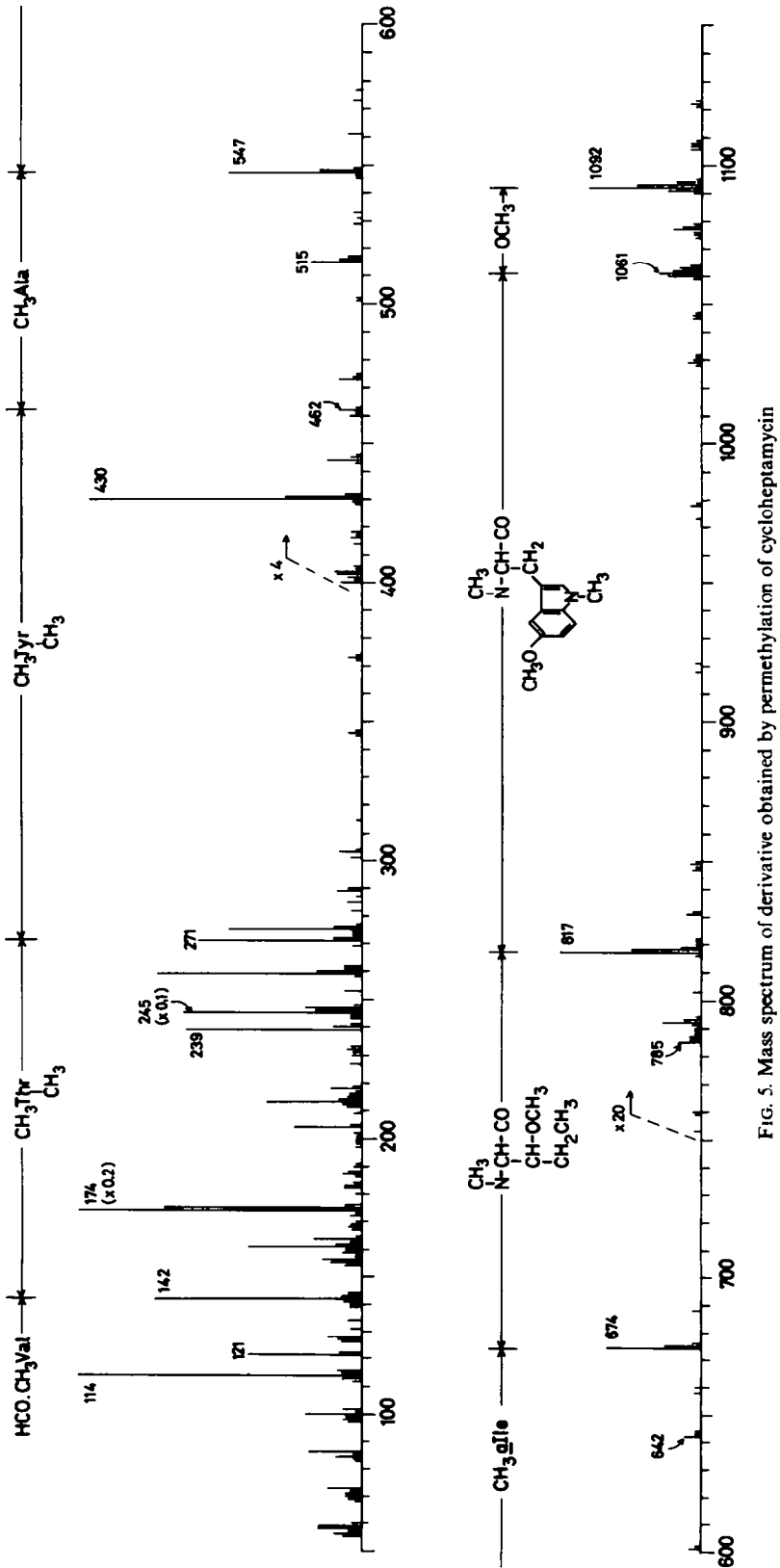


FIG. 5. Mass spectrum of derivative obtained by permethylation of cycloheptamycin

Permethylation of cycloheptamycin and subsequent examination by mass spectrometry revealed a derivative of structure **4**. The mass spectrum of this compound is represented in Fig 5, and its interpretation follows. Mass spectral fragmentation of such a permethylated peptide derivative occurs predominantly at any of the CO—NMe bonds,[17] as indicated, for example, by vertical dashed lines in structure **4**. The number shown to the left of each dashed line is the mass of the N-terminus, including all amino acid residues up to the point of fragmentation. Each of these masses is visible as a major peak in the mass spectrum. Mass differences between successive major peaks represent the masses of corresponding residues in sequential order.

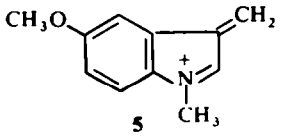
142	271	462	547	674	817	1061	MW 1092
HCO.MeVal	MeThr	MeTyr	MeAla	MeIle	β-hydroxyMeNva	5-methoxyMeTrp	OMe
	Me	Me			Me	Me	

4

Other fragmentation processes may lead to additional major peaks. For example, any fragment which contains a methylated threonine residue tends to lose one molecule of methanol, giving a peak 32 mass units lower than the expected sequence peak. In Fig. 5, this type of fragmentation accounts for the peaks at *m/e* 239, 430, 515, 642, and 785.

In the spectrum of Fig. 5, a molecular ion for the methylated cycloheptamycin derivative was found at *m/e* 1092, accompanied by an ion of mass 1061 due to loss of OMe from the C-terminal methyl ester.

A difference of 244 mass units between *m/e* 1061 and the next "sequence-determining"[17] peak toward lower mass, *m/e* 817, is the mass of the C-terminal residue, and in fact corresponds to the mass of an N,N'-dimethyl derivative of the methoxytryptophane which was suggested above by UV data. Confirmation of the methylated residue was provided by additional mass spectral peaks at *m/e* 174 and *m/e* 245, resulting from fragments **5** and **6**, respectively.



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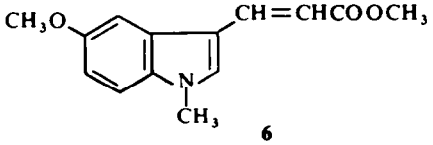

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When deuteromethyliodide was used for permethylation of cycloheptamycin, a labelled derivative was obtained which gave the mass spectrum shown in Fig 6. The N,N'-dimethylmethoxytryptophane of this derivative had a mass of 247 (*m/e* 838 to *m/e* 1085), as compared with 244 in the unlabelled compound. From this it was clear that only one CD_3 group had been introduced, and the other N-Me group (N–C$\underline{H}_3$) must have been present before the methylation reaction. Since deutero-

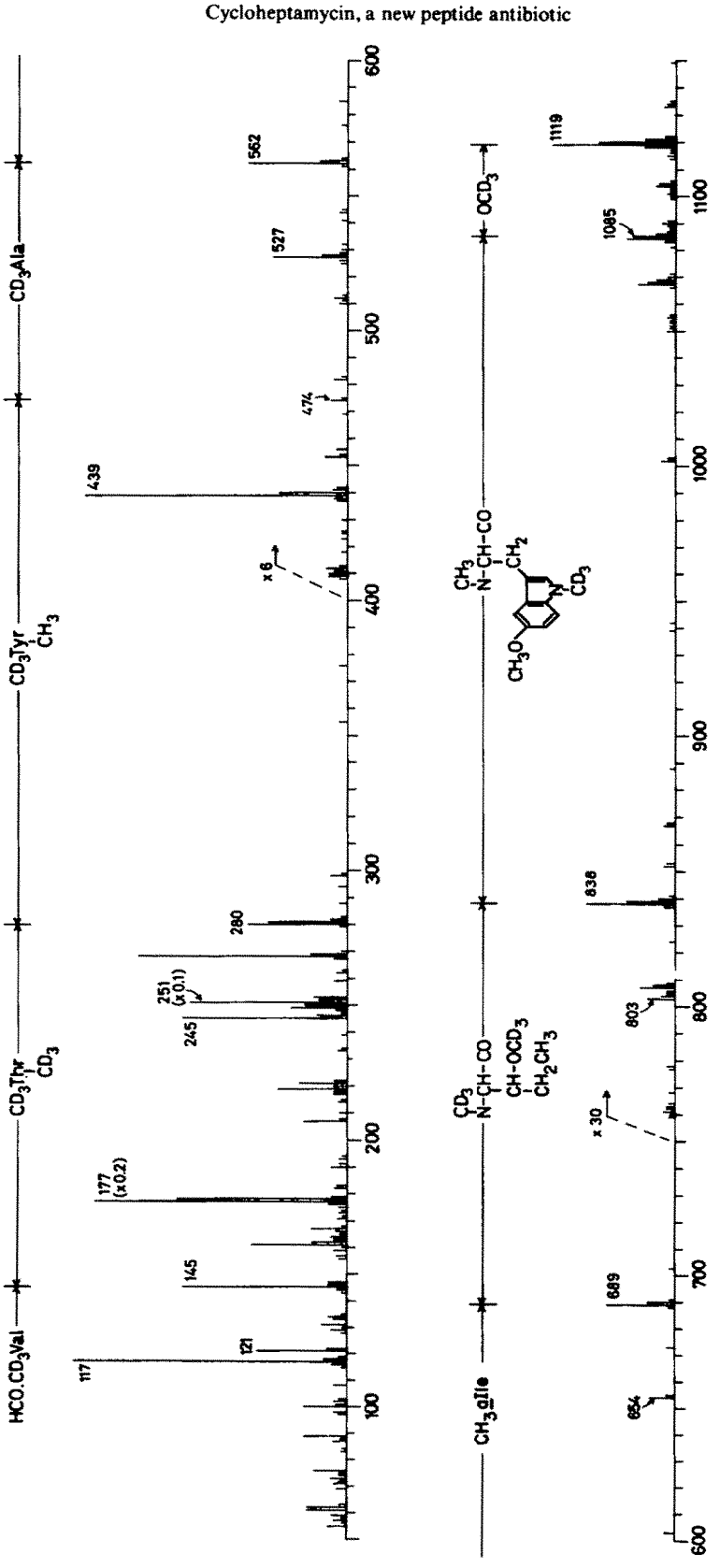


FIG. 6. Mass spectrum of derivative obtained by perdeuteromethylation of cycloheptamycin

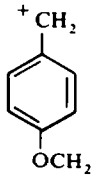
methylation caused a shift of the *m/e* 174 peak (Fig 5) to *m/e* 177 (Fig 6), the labelled Me group was contained within fragment **5**, and present, therefore, at the indole nitrogen. Consequently, cycloheptamycin must possess a free and methylatable indole N—H, and the C-terminal residue of the antibiotic is therefore N-methyl-5-methoxytryptophane.

Working progressively toward lower mass in the spectrum of Fig 5, the next residue of the methylated derivative had a mass of 143 (*m/e* 817 minus *m/e* 674) and contained two Me groups which had been introduced by the permethylation reaction, as demonstrated by the use of CD_3I (compare Figs 5 and 6). These data implied that this was the "unidentified" residue observed in the Moore Stein analysis, and furthermore suggested a hydroxyvaline isomer which was subsequently identified as β-hydroxynorvaline (see above).

The next amino acid residue in the cycloheptamycin sequence had a mass of 127 after methylation with *either* CH_3I or CD_3I. This was, therefore, an N-methylamino acid (isomeric with or identical to N-methylleucine) which had not been detected by Moore Stein analysis. Identification as N-methyl*allo*isoleucine was made as discussed above.

The interval between peaks *m/e* 547 and *m/e* 674 of Fig 5 corresponds to the mass of N-methylalanine (85) which established the location of the alanine residue in cycloheptamycin.

In the mass spectrum of the cycloheptamycin perdeuteromethyl derivative (Fig 6), a peak at *m/e* 121 was due to fragment **7**, originating from a methylated tyrosine residue. To account for the $C\underline{H}_3O$ observed, rather than CD_3O which would have resulted from perdeuteromethylation of a normal tyrosine residue, cycloheptamycin must contain O-methyltyrosine. The position of this residue was established by the peaks *m/e* 271 and *m/e* 462 (Fig 5).



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The "sequence-determining" peak of lowest mass in the spectrum of Fig 5 was found at *m/e* 142, which determined the position of the threonine residue. There is also an intense *m/e* 114 peak, accompanied by a metastable peak corresponding to the transition *m/e* 142 → *m/e* 114, due to loss of CO from the *m/e* 142 fragment. In spectra of permethylated peptide derivatives, only cleavage at the *first* peptide bond is accompanied by such a partial loss of CO, and the observation of a pair of peaks which differ by 28 mass units may help to locate rapidly the first of the "sequence-determining" peaks. Thus, 142 is the mass of the first methylated amino acid residue, together with the original N-terminal blocking group. Of the amino acids known to be present in cycloheptamycin, valine remained to be located, and therefore must be the N-terminal residue.

The difference between the mass of methylvaline (113) and the first sequence peak

(*m/e* 142) is 29 mass units, corresponding to an N-terminal formyl group. The entire sequence of cycloheptamycin was thus established, but some questions remained as to its structure.

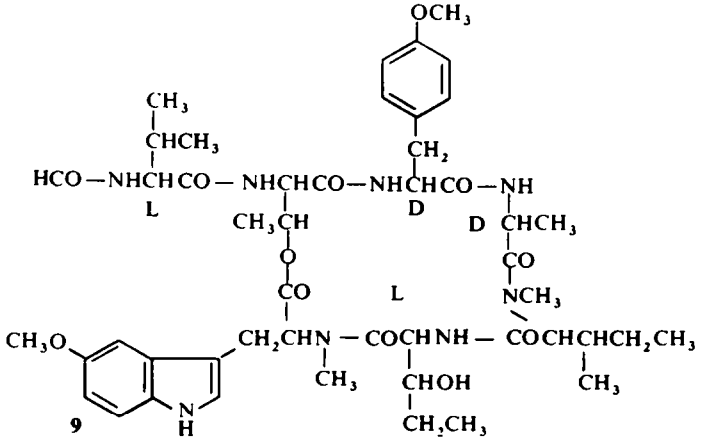
During the methylation procedure, all N— and O—Me groups of a compound remain unchanged, but esters or lactones may become hydrolyzed by the basic reaction conditions, appearing later as methyl esters or ethers. For cycloheptamycin, such a hydrolysis product, intermediate between the antibiotic and the permethyl derivative, must have structure **8**.

HCO.Val.Thr.Tyr.Ala.Me*a*lle.β-hydroxyNva.5-methoxyMeTrp.OH
|
Me

8

It seemed likely that cycloheptamycin contained a lactone, formed by the C-terminal acid group and one of the two hydroxyl functions of structure **8**. This was supported by infrared absorption at 1735 cm^{-1}, and by the formation of a mono-acetate (NMR singlet at $\delta = 2{\cdot}05$) when the antibiotic was acetylated with acetic anhydride in pyridine. This hypothesis was confirmed by the following experiment which also determined that the lactone involved the threonine residue.

Cycloheptamycin was treated with diazomethane in the presence of boron trifluoride—conditions sufficient to form a methyl ether of the free OH group, leaving the lactone intact. Subsequent permethylation with deuteromethyliodide caused hydrolysis of the lactone, and introduced a labelled group at all remaining methylatable positions, including the lactone OH. A mass spectrum of the resulting product was similar to that of Fig 6, including peaks at *m/e* 145, 280, 439, 562, and 689. However, the next sequence peak was found at *m/e* 835, indicative of an unlabelled methyl ether ($OC\underline{H}_3$) in the sixth residue, whereas the fully labelled derivative produced an equivalent peak at *m/e* 838 (Fig 6). Therefore, the hydroxynorvaline residue of cycloheptamycin contains a free OH group which may be methylated by diazomethane, and the threonine OH group is blocked and may be methylated by methyl iodide only after lactone hydrolysis.



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In agreement with all available data, cycloheptamycin is thus represented by structure **9**, which is characterized by several unusual features. Thus, neither N-methyl-5-methoxytryptophane nor β-hydroxynorvaline have previously been detected in natural products. O-methyl-L-tyrosine is a constituent of the antibiotic puromycin,[18] but to the best of our knowledge the D-isomer has not previously been reported.

EXPERIMENTAL

All m.ps are corrected. Optical rotations were measured in $CHCl_3$ ($c = 1$) and UV spectra in 96% EtOH soln. The IR spectra were obtained with a NaCl prism and the NMR spectra with a Varian A-60A (60 Mc) spectrometer, $CDCl_3$ being used as solvent, unless otherwise stated. The line positions are given in δ-values and with TMS as internal reference. For TLC silica gel HF_{254} (Merck) was used as adsorbent. The plates, 250 μ thick, were dried at room temp. For PLC 2 mm plates were used. Microanalyses were performed in the microanalytical laboratory of Leo Pharmaceutical Products by Mr. G. Cornali and Mr. W. Egger. The amino acid analyses were performed at The Danish Institute of Protein Chemistry, Copenhagen. Mass spectra were determined with an A.E.I. model MS 9 mass spectrometer, with a source and sample temperature of 300°.

Isolation and purification of cycloheptamycin

Clarified fermentation broth from Streptomyces MZ 1158 (275 l.) was adjusted to pH 5 and extracted with EtOAc (75 l.). After concentration of the extract to about 1 l., it was washed with water (100 ml) and evaporated *in vacuo* to a brown sirup (35 g). Trituration with ether gave a dark ppt which was filtered off, washed with ether and dried to yield 6·5 g coffee-brown powder. The product was dissolved in $CHCl_3$ (65 ml) and separated from a black insoluble tar. After concentration of the $CHCl_3$ soln to about 25 ml, EtOAc (75 ml) was added. A ppt which separated was removed by washing with water (3 × 25 ml). After drying, the solvent was removed *in vacuo* and the residue triturated with ether to yield 3·0 g tan coloured powder. Further purification was achieved by chromatography on silicic acid (Mallinckrodt, 100 mesh, 150 g). A $CHCl_3$ suspension of the substance was placed on top of the dry-packed column and eluted with EtOAc. The first 3 fractions (each 50 ml) contained the desired substance. Evaporation of the solvent and treatment of the residue with ether gave 1·6 g of a slightly tan-coloured product, which was dissolved in hot EtOH (16 ml) and left at +5° for 3 h. The crystals which formed were filtered off, washed with cold EtOH and ether to yield 1·2 g yellowish white crystals, m.p. 256–258° dec (occasionally the substance melted at 212–214° with subsequent solidification and m.p. 256–258°). Further recrystallizations did not raise the m.p.; $[\alpha]_D^{20}$ +37° ($c = 1$, $CHCl_3$); UV: λ_{max}^{EtOH} 276·5 mμ (ε 7·550), 282 mμ (ε 6·800), 296·5 mμ (ε 4·800) and 308 mμ (ε 3·500); NMR cf. Fig 2. (Found: C, 60·54; H, 7·25; N, 11·74. Calc. for $C_{48}H_{68}N_8O_{12}$: C, 60·74; H, 7·22; N, 11·81%).

Cycloheptamycin is readily soluble in chloroform and acetone, sparingly soluble in MeOH and EtOH, and practically insoluble in water, ether and hexane.

With vanillin in conc HCl it gives a blue colour and with cinnamaldehyde an orange colour. The latter reagent (cinnamaldehyde-ethanol-conc HCl (1:5:5)) is useful for detection of cycloheptamycin on TLC.

By-products. The later EtOAc fractions from the chromatography on silicic acid contained several compounds, which were best detected by TLC (solvent system: CH_2Cl_2–MeOH (9:1)) by spraying with chlorine-tolidine reagent. Separations were performed by dry-column chromatography combined with PLC. In this manner the following compounds were isolated.

L-Leucyl-L-proline, colourless crystals from EtOAc, m.p. 159–163° $[\alpha]_D^{20}$ −126·4 ($c = 1$ MeOH). Lit.[2] m.p. 160–161 $[\alpha]_D^{20}$ −133 (MeOH). (Found: C, 62·81; H, 8·67; N, 13·29. Calc. for $C_{11}H_{18}N_2O_2$: C, 62·83; H, 8·63; N, 13·32%); The IR spectrum (Nujol) was identical with the reported spectrum for L-leucyl-L-proline.

N-Acetyltyramine, light tan crystals from water, m.p. 129·5–130°. Lit.[1] 134–135°. The UV and IR spectra (KBr) were identical with those of an authentic sample. (Found: C, 66·90; H, 7·30; N, 7·78. Calc. for $C_{10}H_{13}NO_2$: C, 67·02; H, 7·31; N, 7·82%).

Acetyltryptamine, was obtained as a yellow oil, which only partly crystallized. The behaviour of the compound in TLC was identical with that of authentic acetyltryptamine (m.p. 78°), and the IR spectra ($CHCl_3$) were indistinguishable.

Isolation of cycloheptamycin and valinomycin from the mycelium of Streptomyces MZ 1158

The wet mycelium (appr. 10 l.) from the aforementioned fermentation was suspended in acetone (10 l.) and stirred for 1 hr. After filtration through dicalite and washing with acetone, the filtercake was refluxed with acetone (5 l.) for 16 hr. Filtration and washing with acetone left 2·5 kg dried mycelium. The acetone extracts and washings were combined and saturated with NaCl. The supernatant was separated and the aqueous phase extracted with EtOAc (2 × 2 l.).

The combined organic phases was concentrated *in vacuo* until a mixture of oil and water remained. The oil was extracted with EtOAc and the extract washed with 3% $NaHCO_3$ aq followed by water. After drying, the solvent was removed *in vacuo* to give 65 g of a light brown oil. The oil was dissolved in light petroleum (b.p. < 50°, 500 ml) and left in the refrigerator at +5° night over. A ppt which separated was filtered off and washed with light petroleum to give 4·0 light brown powder. Treatment of this with ether left 1·7 g undissolved. Chromatography of this product on silicic acid and purification as described above afforded 600 mg cycloheptamycin m.p. 256–258° dec, identical with the product obtained from the clarified fermentation broth.

The light petroleum filtrate was concentrated to about 100 ml and poured onto a column of faintly acidic alumina (300 g). Elution with light petroleum (3 l.) gave an oil (15 g) which crystallized upon standing. The crystals were isolated and washed with light petroleum to yield 1·4 g valinomycin, m.p. 184–186°, $[\alpha]_D^{20}$ +30° (*c* = 1, benzene), Lit.[4] m.p. 187° $[\alpha]_D^{20}$ +32·8° (*c* = 1, benzene). (Found: C, 58·74; H, 8·20; N, 7·26. Calc. for $[C_{18}H_{30}N_2O_6]_3$: C, 58·36; H, 8·16; N, 7·56%); The NMR spectrum (DMSO) is identical with that reported for valinomycin.[19,20]

Permethylation[21] *of cycloheptamycin.* Cycloheptamycin (3 mg) was dissolved in 0·2 ml DMF. Sodium hydride oil suspension (50 mg) was rinsed 3 times with ether, then added to the peptide soln, followed by 0·1 ml MeI. After 1 hr at room temp with continuous stirring, the mixture was added to water and the product was extracted with $CHCl_3$, dried with Na_2SO_4, and evaporated to dryness. A mass spectrum of this product is shown in Fig. 5. When CD_3I was used, a product was obtained which gave the mass spectrum of Fig. 6.

Methylation of cycloheptamycin. Cycloheptamycin (2 mg) was dissolved in dichloromethane (1 ml) containing diazomethane. Dichloromethane (20 µl) containing BF_3–Et_2O (0·2 µl) B was added at room temp, and after 5 min the soln was evaporated to dryness. The product was permethylated with CD_3I by the procedure described above, and a mass spectrum of the resulting derivative is described in the text.

Acid hydrolysis of cycloheptamycin and TLC of the hydrolysate. A suspension of cycloheptamycin (10 mg) in 6N HCl (2 ml) was refluxed for 24 hr. After evaporation to dryness *in vacuo*, the residue was dissolved in water and treated with Amberlite IR45 until a pH of about 6 was reached. The resin was removed and the filtrate taken to dryness, whereafter the residue was dissolved in 1 ml water.

Identification of β*-hydroxynorvaline.* Each of the five hydroxyvaline isomers (1·3 mg) to be tested was dissolved in 1 ml of a 0·01 M aqueous soln of alanine, valine, threonine and tyrosine, in order to create conditions comparable to that of the hydrolysate. Samples (1 µl) were applied to 20 × 20 cm plates, which were subjected to 2-dimensional development, first in the solvent system BuOH–AcOH–water (2:1:1), followed by the system phenol–water (3:1). A similar set of plates were run in the solvent systems $CHCl_3$–MeOH–17% NH_4OH (2:2:1) and phenol–water (3:1). The amino acids were visualized by spraying with ninhydrine-cupric nitrate reagent. The hydroxyvalines were positioned between alanine and valine, proximate to alanine. Comparison of the plates permitted safe exclusion of all but one of the tested hydroxyvalines, namely β-hydroxynorvaline.

Continuous development of thin-layer chromatograms. In order to achieve a satisfactory separation by TLC, on the one hand of threonine and *allo*threonine, on the other of the four N-methyl-leucine isomers, a simple continuous development technique was used.

A strip of filterpaper (10 × 48 cm) was folded thrice and placed at the top of the plate (10 × 20 cm) with 6 layers of paper at the back of the plate and 2 layers at the coated side. Good contact with the adsorbent was ensured by placing a piece of glass (5 × 10 cm) over the paper. The assembly was held together with two clamps, and developed vertically for 6–8 h. Samples were applied in volumes of 1 µl, and each sample was applied at least twice to the same plate, in order to eliminate possible errors due to irregularities in the solvent front. After spraying with ninhydrine-cupric nitrate reagent, the spots were characterized by the distance moved.

Identification of threonine. Separation of threonine and *allo*threonine as well as of threonine and alanine in the hydrolysate was achieved in the solvent system EtOH–$CHCl_3$–water–25% NH_4OH (45:42:10:3) by means of the above technique. Distances measured: *Allo*threonine 45 mm, threonine 41 mm and the spot in the hydrolysate 41 mm.

Identification of N-*methyl*allo*isoleucine*. Continuous development in the solvent system BuOH–AcOH–water (8:1:1) readily separated N-methylnorleucine (44 mm) and N-methylleucine (43 mm) from N-methylisoleucine (34 mm). The spot in the hydrolysate moved 34 mm.

The separation of N-methylisoleucine and N-methyl*allo*isoleucine was effected by continuous development in the solvent system i-PrOH–$CHCl_3$–25% NH_4OH (68:68:15). Due to interfering amino acids in the hydrolysate this was replaced by the hydrolysates treated with either L- or D-amino acid oxidase (*vide infra*). Distances measured: N-methylisoleucine 28 mm, N-methyl*allo*isoleucine 31 mm, and the spots in the amino acid oxidase treated hydrolysates both 31 mm.

D,L-Erythro-β-*hydroxynorvaline*. The procedure described by Hellmann[8a] was used with minor modifications. A mixture of acetaminomalonic acid monoethyl ester (30·2 g, 0·16 mole), freshly distilled propionaldehyde (18·5 g, 0·32 mole) and Et_3N (22·6 ml, 0·15 mole) was left for 3 days at room temp. EtOAc (100 ml) was added, and an insoluble ppt filtered off (5·8 g). This product was presumably the Et_3N-salt of the unreacted mono-acid since acid hydrolysis yielded glycine. The filtrate was concentrated to a yellow syrup *in vacuo*, conc HCl (80 ml) was added, and the mixture refluxed for 2 hr. After evaporation to dryness, the residue was extracted with water. An insoluble dark tar was removed and the yellow filtrate was stirred with Amberlite IR45 until a pH of 5 was reached. After removal of the resin the filtrate was concentrated to about 30 ml. The separated crystals were collected, washed with EtOH–water (1:1) and EtOH to yield 8·6 g m.p. 236° (dec). Concentration of the filtrate and precipitation with EtOH gave a mixture of β-hydroxynorvaline and glycine (7 g) from which a further crop of β-hydroxynorvaline (2·5 g, m.p. 238°) could be obtained by recrystallization from water–EtOH. Further recrystallizations from water–EtOH raised the m.p. to 244° (dec), reported: m.p. 236°,[8b] m.p. 245–246°,[8c] m.p. 257–259°.[11a] (Found: C, 44·99; H, 8·29; N, 10·49. Calc. for $C_5H_{11}NO_3$: C, 45·10; H, 8·33; N, 10·52%).

N-*Benzoyl*-D,L-erythro-β-*hydroxynorvaline methyl ester* was prepared from the N-benzoylated amino acid (m.p. 180–181°[8b, 11a]) by esterification with diazomethane in ether suspension. The ester crystallized from ether, m.p. 115·5–116°, reported m.p. 114°.[8b] (Found: C, 62·03; H, 6·82; N, 5·57. Calc. for $C_{13}H_{17}NO_4$: C, 62·14; H, 6·82; N, 5·57%).

D,L-trans-2-*phenyl*-4-*carbomethoxy*-5-*ethyl*-Δ^2-*oxazoline* (**3b**). N-Benzoyl-D,L-*erythro*-β-hydroxynorvaline methyl ester (8 g) was cooled to about −10°. Purified $SOCl_2$ (24 ml) was added portionwise, the temp being kept below 0°. When the addition was complete, the mixture was allowed to warm to room temp. After 1 hr at room temp, the $SOCl_2$ was removed *in vacuo* and the resulting yellow oil was dissolved in $CHCl_3$. After treatment with an excess of $KHCO_3$ aq, the organic layer was washed with water, dried and evaporated *in vacuo* to yield 7·75 g of a pale yellow oil. A sample was distilled *in vacuo* (0·05 mm) for analytical purposes. (Found: C, 66·93; H, 6·49; N, 5·81. Calc. for $C_{13}H_{15}NO_3$: C, 66·93; H, 6·48; N, 6·01%).

D,L-Threo-β-*hydroxynorvaline*. Acid hydrolysis of the above oxazoline (**3b**) (7·5 g) in boiling 6N HCl (60 ml) for 2 hr, gave, after evaporation to dryness, treatment with Amberlite IR45 in aqueous soln, and renewed evaporation, 3·6 g m.p. 220–221° dec). Recrystallization from water–EtOH did not raise the m.p., reported: m.p. 218°,[8b] m.p. 217–218°,[8c] m.p. 227–228°.[11a] (Found: C, 45·09; H, 8·28; N, 10·38. Calc. for $C_5H_{11}NO_3$: C, 45·10; H, 8·33; N, 10·52%).

N-*Benzoyl*-D,L-threo-β-*hydroxynorvaline methyl ester* was prepared as described via the N-benzoylated amino acid, m.p. 151–153°, reported m.p. 149°[8b] and m.p. 154–156°.[11a] It was obtained as a colourless oil, which could not be induced to crystallize.

D,L-cis-2-*Phenyl*-4-*carbomethoxy*-5-*ethyl*-Δ^2-*oxazoline* (**3a**) was prepared exactly as described for the *trans*-oxazoline. A sample was distilled *in vacuo* at 0·05 mm. (Found: C, 66·95; H, 6·38; N, 5·86. Calc. for $C_{13}H_{15}NO_3$: C, 66·93; H, 6·48; N, 6·01%). Acid hydrolysis of the *cis*-oxazoline gave, as expected, D,L-*erythro*-β-hydroxynorvaline, m.p. 244°.

N-*Methylleucine isomers*

The N-methylation of the four leucine isomers was carried out essentially as outlined by Lederer.[13] An illustrative example is given below. N-Acetyl-D-*allo*isoleucine (900 mg), dry Ag_2O (4·5 g), dry DMF (9 ml) and MeI (6 ml) were stirred magnetically for 16 hr in a stoppered flask at 50°. Water (50 ml) and ether (50 ml) were added and the mixture stirred for 30 min, whereafter the Ag salts were removed by filtration. The ether layer was separated and the water phase extracted twice with ether. The combined ether solns were washed with water, 0·1N HCl and water. After drying, the solvent was removed to yield an almost colourless oil (800 mg). Acid hydrolysis in 6N HCl for 2 hr gave, after evaporation to dryness, treatment with Amberlite IR45 in aqueous soln and repeated evaporation to dryness, 510 mg of almost pure crystals (TLC). Recrystallization from water–EtOH gave a pure product, which did not melt but sublimed when heated above 250°.

N-Methyl-D,L-norleucine, N-methyl-L-leucine and N-methyl-L-isoleucine were prepared in a similar way. None of the compounds exhibited a m.p. but sublimed when heated above 250°. In all cases correct microanalyses, deviating less than 0·25% from the theoretical values, were obtained.

Enzymatic oxidations

Materials. The enzymes were obtained from Boehringer und Soehne, G.m.b.H. Mannheim, Western Germany. The specific activity of the D-amino acid oxidase from pig kidney and the L-amino acid oxidase from Crotalus terr. terr. were approximately 15 and 5 units/mg, respectively. Catalase, from beef liver, contained 39000 units/mg and peroxidase, from horse radish, 180 units/mg. The buffers were prepared as follows: Sodium pyrophosphate buffer pH 8·2: 50 ml of 0·1 M $Na_4P_2O_4$ were mixed with 23·7 ml of 0·1N HCl. Tris buffer, pH 7·7: 25 ml of 0·2 M Tris were mixed with 36·7 ml of 0·1N HCl and diluted to 100 ml with water.

Procedure. Cycloheptamycin (30 mg) was hydrolyzed as described before. An almost neutral soln, containing 10 mg/ml, was prepared and divided into three portions, one of which was subjected to the action of DAO, another to LAO, while the remaining part was used as control.

The enzymatic oxidations were carried out in a modified Warburg apparatus at 37°C with the use of air as gas phase essentially as described by Ishi and Witkop.[22]

D-Amino acid oxidase reaction. 1 ml of hydrolysate (10 mg/ml), 1 ml of pyrophosphate buffer, 10 µl of catalase soln (3900 u/mh) and 50 µl of NADH soln (10 mg/ml) were incubated at 37°. After equilibration 10 µl of D-amino acid oxidase (75 u/ml) was added and incubation continued for 16 hr.

L-Amino acid oxidase reaction. 1 ml of hydrolysate (10 mg/ml), 1 ml of Tris buffer and 10 µl of peroxidase (10 mg/ml) were incubated at 37°. After equilibration, 100 µl of L-amino acid oxidase (5 u/ml) was added and incubation continued for 16 hr.

The amino acids remaining in the hydrolysates after the enzymatic oxidations were assayed in the amino acid analyzer and subjected to two-dimensional TLC in the systems: butanol–acetic acid–water (2 : 1 : 1) and phenol–water (3 : 1). The results of the Moore Stein analyses appear from Fig. 4. The chromatograms of the DAO-treated hydrolysate revealed the presence of the following amino acids: valine, threonine, N-methyl*allo*isoleucine and β-hydroxynorvaline. The LAO-treated hydrolysate contained the following amino acids: threonine, tyrosine, alanine, and N-methyl*allo*isoleucine.

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