LIEBIGS ANNALEN DER CHEMIE

HERAUSGEGEBEN VON DER
GESELLSCHAFT DEUTSCHER CHEMIKER

JAHRGANG 1985 · HEFT 5 · SEITE 877–1098

Dieses Heft wurde am 13. Mai 1985 ausgegeben

VCH Verlagsgesellschaft

Amiclenomycin Peptides – Isolation and Structure Elucidation of New Biotin Antimetabolites

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Received June 12, 1984

Six peptide antibiotics were isolated from the culture filtrate of *Streptomyces venezuelae* Tü 2460. The 4-amino-2,5-cyclohexadienyl ring of the amino acid amiclenomycin (Acm, **7**) was found to be the essential structural element of the three dipeptides L-MeIle-L-Acm (**1**, stravidin S_3), L-Ile-L-Acm (**2**), and L-MeVal-L-Acm (**3**, stravidin S_2) as well as the three tripeptides L-MeIle-L-Acm-L-Gln (**4**), L-Ile-L-Acm-L-Gln (**5**) and L-Val-L-Acm-L-Gln (**6**). In addition L-amiclenomycin (**7**) and L-Acm-L-Gln (**8**) were obtained by enzymatic cleavage with pronase P. All eight antibiotics inhibit the growth of gram-negative bacteria by blocking the biotin biosynthesis. A synergistic enhancement of the antibiotic action was found by a polypeptide L (**9**) also isolated from *S. venezuelae* Tü 2460. Further screening[*)] revealed, that the *S. venezuelae* strain Tü 2605 produces the antibiotic L-MeIle-L-Acm-L-Glu (**10**).

Amiclenomycin-Peptide – Isolierung und Strukturaufklärung neuer Biotin-Antimetaboliten

Sechs Peptidantibiotika wurden aus dem Kulturfiltrat von *Streptomyces venezuelae* Tü 2460 isoliert. Der 4-Amino-2,5-cyclohexadienyl-Ring der Aminosäure Amiclenomycin (Acm, **7**) wurde als essentielles Strukturelement in den drei Dipeptiden L-MeIle-L-Acm (**1**, Stravidin S_3), L-Ile-L-Acm (**2**) und L-MeVal-L-Acm (**3**, Stravidin S_2) sowie in den drei Tripeptiden

[*)] Supplemented on correction (Nov. 7, 1985).

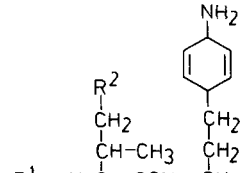
Liebigs Ann. Chem. **1985**, 877–892

0170–2041/85/0505–0877 $ 02.50/0

L-MeIle-L-Acm-L-Gln (**4**), L-Ile-L-Acm-L-Gln (**5**) und L-Val-L-Acm-L-Gln (**6**) ermittelt. Zudem wurden L-Amiclenomycin (**7**) und L-Acm-L-Gln (**8**) durch enzymatische Spaltung mit Pronase P erhalten. Alle acht Antibiotika hemmen das Wachstum gramnegativer Bakterien durch Blockade der Biotinbiosynthese. Eine synergistische Verstärkung der Antibiotikawirkung wurde durch ein ebenfalls aus *S. venezuelae* Tü 2460 isoliertes Polypeptid L (**9**) erzielt. Eine weitere Untersuchung[*] ergab, daß der *S.-venezuelae*-Stamm Tü 2605 das Antibiotikum L-MeIle-L-Glu (**10**) produziert.

The strain *Streptomyces venezuelae* Tü 2460 was found to produce an antibiotic activity against gram-negative bacteria. This activity was found to be reversed by addition of biotin. In the following we describe the isolation and structure elucidation of five new and three known antibiotics from the new strain of *S. venezuelae* Tü 2460. These biotin antimetabolites contain the previously described amino acid amiclenomycin (Acm)[1] which has a 4-amino-2,5-cyclohexadienyl ring in the side chain (Scheme 1).

Scheme 1. Structures of amiclenomycin-containing biotin antimetabolites **1**–**6** isolated from *S. venezuelae* Tü 2460



			R^1	R^2	R^3
1	*N*-Methyl-L-isoleucyl-L-amiclenomycin	MeIle-Acm	CH_3	CH_3	OH
2	L-Isoleucyl-L-amiclenomycin	Ile-Acm	H	CH_3	OH
3	*N*-Methyl-L-valyl-L-amiclenomycin	MeVal-Acm	CH_3	H	OH
4	*N*-Methyl-L-isoleucyl-L-amiclenomycyl-L-glutamine	MeIle-Acm-Gln	CH_3	CH_3	Gln
5	L-Isoleucyl-L-amiclenomycyl-L-glutamine	Ile-Acm-Gln	H	CH_3	Gln
6	L-Valyl-L-amiclenomycyl-L-glutamine	Val-Acm-Gln	H	H	Gln

Spectroscopic and chromatographic analyses showed that the biotin antimetabolites **1**–**6** are di- and tripeptides which have the essential amino acid amiclenomycin in common. This unusual amino acid has been isolated by *Okami* et al.[1] from the culture filtrate of *Streptomyces lavendulae.* The antimetabolites **1** and **3** are the known antibiotics stravidin S_3 and S_2[2,3], whereas the antibiotics **2, 4, 5,** and **6** are new antimetabolites.

During the isolation of the stravidines S_2 and S_3 a larger polypeptide of molecular mass 60000 was found, this so-called streptavidin[4,5] has complexing activities for biotin. We also observed a higher polypeptide L (**9**) in the culture filtrate of *Streptomyces venezuelae* Tü 2460, which was isolated as shown in Scheme 2. Solubility, amino acid composition, and molecular mass indicate clearly that our polypeptide L differs completely from the streptavidin described in the literature.

[*] Supplemented on correction (Nov. 7, 1985).

The new polypeptide L (**9**) can enhance synergistically the activities of the biotin antimetabolites **1**–**8**.

Fermentation and Isolation of the Antibiotics

At first we used a complex medium containing malt and meat extract. However, from 1000 l only 50 mg of polar antibiotic mixture could be isolated. Complications by concomitant amino acids and oligopeptides afforded tedious purification steps. Therefore a minimal medium was developed which also lead to large increase in production. During the isolation procedures the detection of the antibiotics was only possible by bioautography and agar diffusion assay.

In small batches the biotin antimetabolites may be enriched by extraction from alkaline aqueous solutions with 1-butanol. Isolation by ion exchange chromatography on CM Sephadex or Dowex 50-X8 gives only very moderate yields. From the culture filtrate of 200-l fermenters repeated adsorption on Amberlite XAD-4 and elution with aqueous methanol proved to be a suitable way for the enrichment of larger amounts of antibiotics. Counter-current distribution in 0.2 N ammonia/1-butanol was found to be most efficient for the purification and fractionation of the polar antibiotics. We also used gel chromatography on Sephadex G-15 and LH-20, and the final purification and separation of the natural analogues was performed by HPLC on reversed amine phases, LiChrosorb-NH_2, or μ-Bondapak-NH_2 (Scheme 2).

The efficiency of the HPLC system used is demonstrated in Fig. 1 (above), which shows a one-run separation of all six closely related active components of the antibiotic mixture and a final purification for two components with base line separation [Fig. 1 (below)].

Spectra and Structure Elucidation of the Antimetabolites

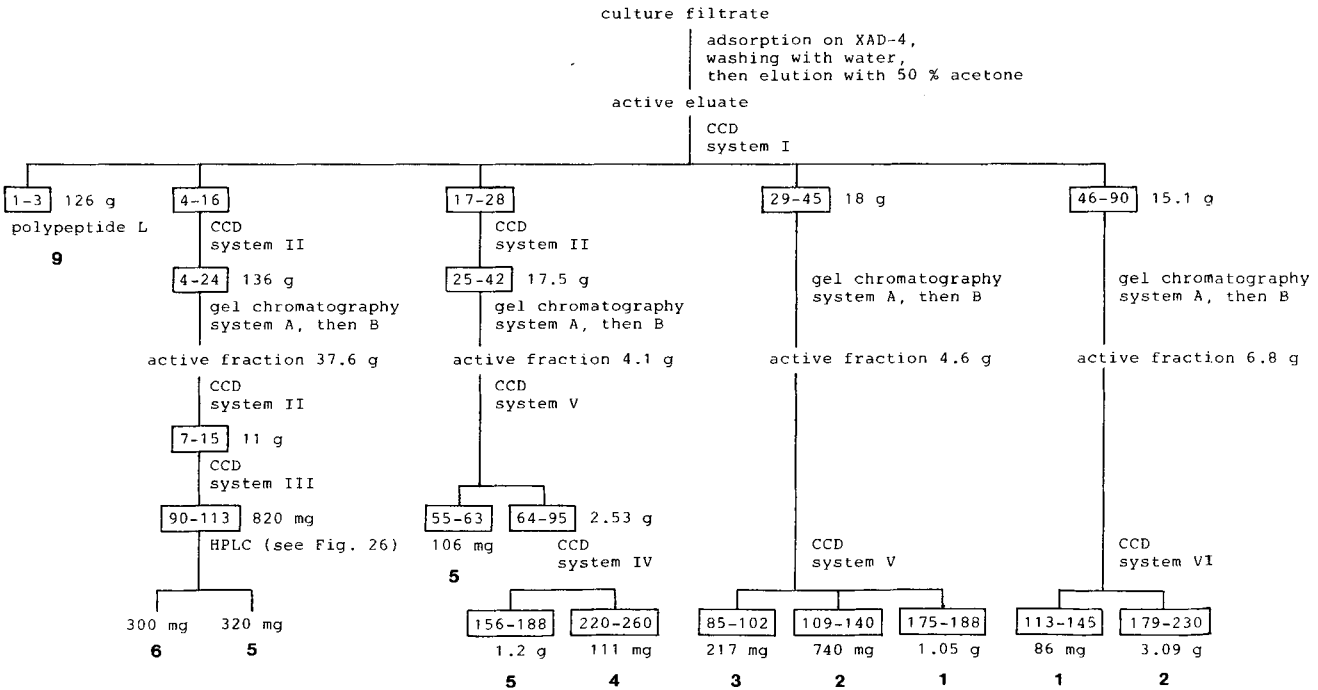
The ^{13}C and ^{1}H NMR spectra of all six components **1**–**6** revealed a common structural component (Tables 2 and 3). The amino acid analyses of the total hydrolysates of all antimetabolites showed L-homophenylalanine [HPhe, (*S*)-2-amino-4-phenylbutanoic acid] and a high ammonia content. The identity of L-HPhe was proved by comparison with the synthetic amino acid using GC-MS, GC on a chiral phase, amino acid analyses, and TLC. According to the NMR data of the native antimetabolites **1**–**6**, homophenylalanine must be a degradation product of an acid-labile component. Homophenylalanine was formed quantitatively in boiling neutral water within about 1.5 hours as found by NMR following the increase of the signals of aromatic (δ = 7.25) and decrease of the signals of olefinic protons (δ = 5.8–6.2). In the same experiment 1 mol of ammonia was released quantitatively. Since peptide amides are stable under these conditions, the elimination of ammonia and aromatization of a symmetrically substituted olefinic ring seemed to occur simultaneously.

Amiclenomycin (7)

From ^{13}C and ^{1}H NMR spectra (double resonance, off resonance, and *J*-modulated spin-echo experiments) we concluded that the amino acid L-2-amino-4-

Scheme 2. Isolation of the amiclenomycin peptides from the culture medium of four 200-l fermenters of *Streptomyces venezuelae* Tü 2460

Chromatographic systems: A = Sephadex G-15, 0.02 N acetic acid; B = Sephadex LH-20, 0.2 N ammonia/methanol (3:7). – Counter-current distribution (CCD) in 0.2 N ammonia/1-butanol: I = 110 elements à 500 ml, 110 steps; II = 105 elements à 50 ml, 150 steps; III = 440 elements à 10 ml, 1600 steps; IV = 440 elements à 10 ml, 1500 steps; V = 440 elements à 10 ml, 660 steps; VI = 440 elements à 10 ml, 800 steps. – HPLC separation: see Fig. 1.



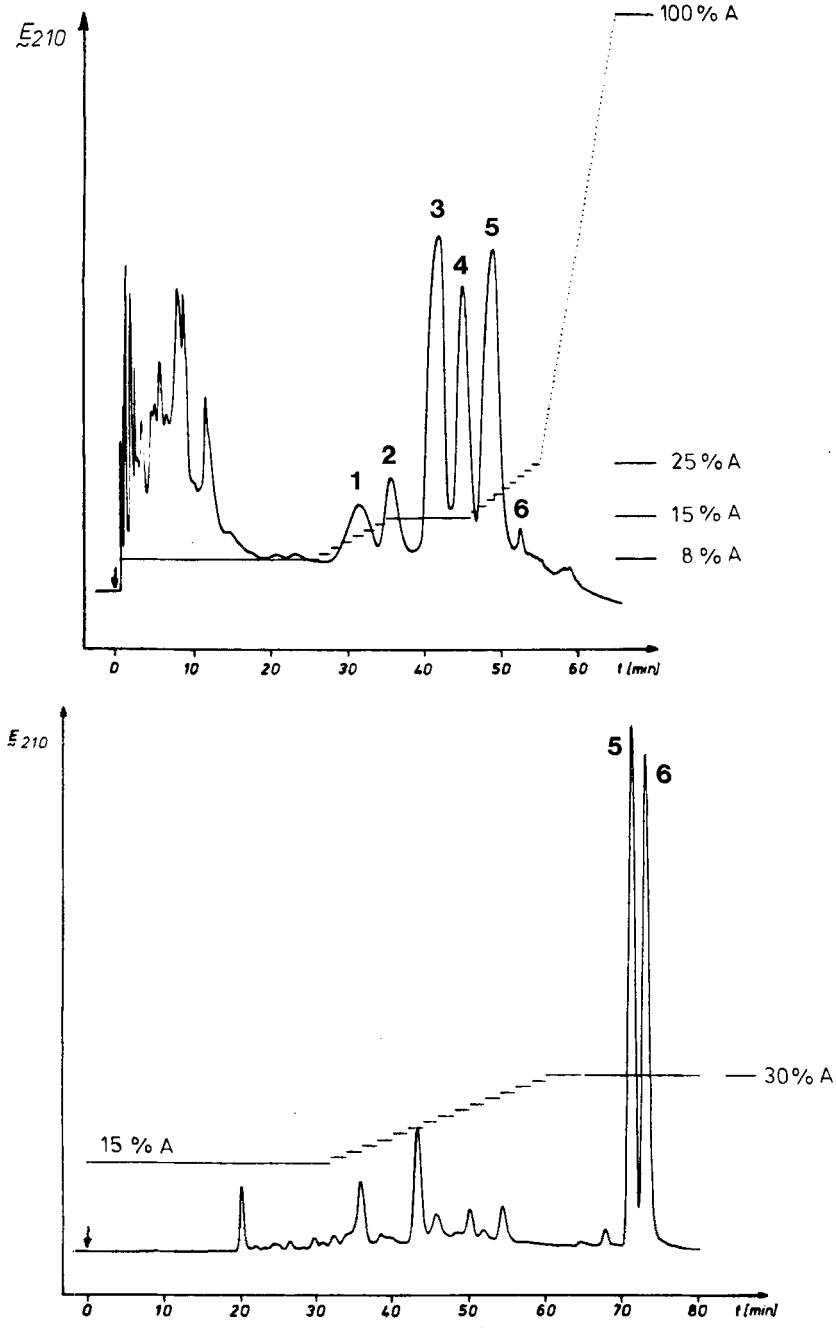


Figure 1. HPLC chromatograms of the separation of the antimetabolites **1**–**6** on LiChrosorb-NH_2 (length 25 cm, diameter 20.5 mm) with gradients of A: 10 mM KH_2PO_4 and B: acetonitrile/water (50:7), 1.5 ml/min, UV detection (210 nm)

(4-amino-2,5-cyclohexadienyl)butanoic acid [amiclenomycin, Acm (7)] must be the common essential structural element of all six antimetabolites (Scheme 1). According to the coupling constant of 7.5 Hz of the η-H signal the ring system is a *trans*-4-amino-2,5-cyclohexadienyl group[6].

N-Methyl-L-isoleucyl-L-amiclenomycin (1)

In addition to Acm, the antimetabolite **1** contains a further nonprotein amino acid as found by GC and GC-MS of pentafluoropropionyl(Pfp)amino acid *n*-propyl(nPr) esters. ^{13}C and ^{1}H NMR spectra of **1** suggested a *N*-methylisoleucine (MeIle) residue.

The structures of Acm and MeIle were confirmed by homo- and heteronuclear double-resonance experiments (Table 4) and by comparison with MeIle derivatives using GC-MS. The L-configuration of Acm was determined *via* GC of Pfp-HPhe-O-nPr on Chirasil-Val[7], whereas the L-configuration of MeIle was assigned *via* GC of the *N-tert*-butylureido-MeIle-*N-tert*-butylamide[8] on the same phase.

The sequences of the antimetabolites were determined by converting the amiclenomycin peptides to homophenylalanine peptides followed by dansyl Edman degradation and two-dimensional TLC on polyamide[9]. The degradation reaction was carried out with phenyl- and methylisothiocyanate, and the antimetabolite **1** was found to contain N-terminal N^{α}-methyl-L-isoleucine.

The FD-MS was in agreement with that of MeIle-Acm: found m/e = 324 $[M + H]^+$ (100%) and 307 $[M + H - NH_3]^+$ (30%). Esterification (diazomethane) and acylation (trifluoroacetic anhydride) of dipeptide **1** gave two volatile derivatives which could be analyzed by GC-MS on a SE 52 quartz capillary. The main peak was that of Tfa-MeIle-Acm(Tfa)-OMe with a longer retention time than the minor peak which was assigned to Tfa-MeIle-HPhe-OMe. In partial hydrolysates analyzed for DL determination (see above) we always found Pfp-MeIle-HPhe-O-nPr. Thus, the antimetabolite **1** is unequivocally N^{α}-methyl-L-isoleucyl-L-2-amino-4-(4-amino-2,5-cyclohexadienyl)butanoic acid (MeIle-Acm).

N-Methyl-L-isoleucyl-L-amiclenomycyl-L-glutamine (4)

Because of its close spectral resemblance to dipeptide **1** the antibiotic **4** was analyzed next. The total hydrolysate contained L-glutamic acid besides MeIle, HPhe and NH_3 according to the amino acid analyses and GC-MS of Pfp/nPr and *N-tert*-butylureido-*N-tert*-butylamide derivatives. The enzymatic cleavage with the peptidase pronase P (method B) yielded MeIle, Acm, and glutamine. Dansyl Edman degradation revealed the tripeptide MeIle-HPhe-Gln. Further proof for the sequence MeIle-Acm-Gln was obtained by comparison with the analogous tripeptide MeIle-Acm-Glu isolated from *Streptomyces venezuelae* Tü 2605 (data see Experimental)*). Furthermore, FD-MS of the nonderivatized antimetabolite **4** and both ^{1}H and ^{13}C NMR spectra were also unequivocally interpretable with N^{α}-methyl-L-isoleucyl-L-2-amino-4-(4-amino-2,5-cyclohexadienyl)butanoyl-L-glutamine (MeIle-Acm-Gln).

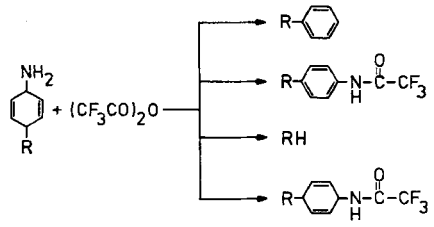
*) Supplemented on correction (Nov. 7, 1984).

N-Methyl-L-valyl-L-amiclenomycin (3)

The total hydrolysate of the antibiotic **3** only revealed HPhe in the amino acid analyzer, whereas *N*-methyl-L-valine (MeVal) and L-HPhe were found by GC-MS on Chirasil-Val.

The FD-MS of non derivatized **3** showed $m/e = 310$ $[M + H]^+$ and 309 M^+ and the typical fragment 292 $[M - NH_3]^+$. The Tfa/OMe derivative of **3** gave four peaks of similar intensities in GC-MS on SE 52. Their origin was correlated with the partial degradation to homophenylalanine, 4-aminohomophenylalanine [HPhe(4′-NH_2)], and homoalanine (HAla) derivatives. The four compounds were identified as Tfa-MeVal-HPhe-OMe, Tfa-MeVal-HPhe(4′-NH-Tfa)-OMe, Tfa-MeVal-HAla-OMe, and possibly Tfa-MeVal-HPhe-OMe or Tfa-MeVal-Acm-(Tfa)-OMe, respectively (no M^+ peaks). These findings suggest the reactions of the cyclohexadienyl system shown in Scheme 3.

Scheme 3



Analogous experiments with **1** revealed mainly compounds corresponding to peaks 1 and 2.

After conversion of antimetabolite **3** to the homophenylalanine peptide (water, 100 °C, 90 min) followed by Edman degradation we obtained N-terminal MeVal. ^{13}C and ^1H NMR spectra (Fig. 2) were in full agreement with those of N^{α}-methyl-L-valyl-L-amiclenomycin (MeVal-Acm). The assignments of the ^{13}C NMR signals were confirmed by off-resonance and spin-echo experiments which also allowed a distinction between two close signals at $\delta = 32.1$ (Acm C-3) and $\delta = 32.4$ (Val C-β).

Antimetabolites 2, 5, and 6

This group of antibiotics contains only the protein amino acids L-Ile, L-Gln, and L-Val besides L-Acm. FD-MS of the antimetabolites revealed the dipeptide Ile-Acm (**2**) and the tripeptides Ile-Acm-Gln (**5**) and Val-Acm-Gln (**6**). The sequence Ile-Acm was compatible with results of GC-MS of the Tfa/OMe derivative of the antimetabolite **2** which showed again four peaks indicating the same reaction pattern as found for **3**. The Tfa/OMe derivatives of **5** and **6** were not volatile enough for GC.

Dansyl Edman degradation was performed again on the corresponding HPhe derivatives and revealed unequivocally the sequences indicated above. ^{13}C and ^1H NMR spectra of **2, 5,** and **6** were in full agreement with these sequences.

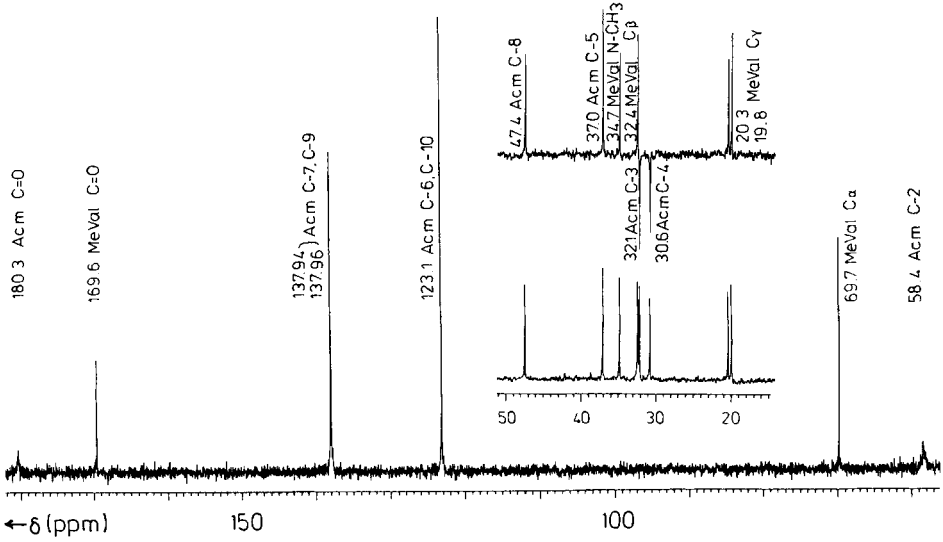


Figure 2. ^{13}C NMR spectrum (100.62 MHz) of MeVal-Acm (**3**), broad-band decoupled and *J*-modulated spin-echo spectrum ($\delta = 15-50$) in D_2O (pH = 7)

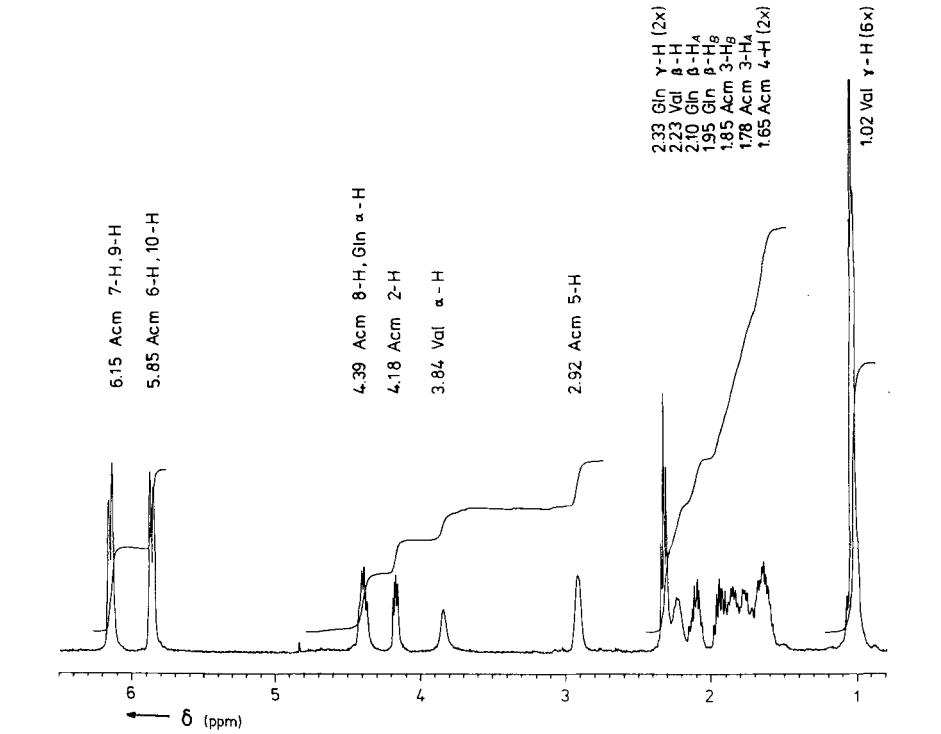


Figure 3. ^1H NMR spectrum (400.13 MHz) of the tripeptide Val-Acm-Gln (**6**) in D_2O (pH = 7)

IR, UV, and CD Spectra

The IR spectra (Table 5) of the antibiotics **1**–**6** show characteristic frequencies of the 1,4-cyclohexadienyl system. The typical bands of amino acids and peptides are found at 3500–3100 (NH), 1740–1600 (amide I), and 1630–1500 cm^{-1} (amide II). The antimetabolites **1**–**6** show in the UV spectra only the absorption maximum at 217 nm of the peptide-carbonyl n → π* transitions. The CD spectra of the dipeptides **1**–**3** with negative Cotton effect at 210–220 nm differ characteristically from those of the tripeptides **4** and **5** (Table 7). The antimetabolite **6** has no intense ellipticities.

Enzymatic Degradation

Different proteolytic activity of the pronase P from *Streptomyces griseus* was observed according to solution conditions: When enzymatic degradation was performed in buffer solution (method A), the Ile–Acm peptide bond in the antimetabolite **2** was cleaved quantitatively. The tripeptides **5** and **6** were cleaved to yield Ile and Val, respectively, and the new dipeptide antimetabolite L-amiclenomycyl-L-glutamine (**8**, Acm-Gln), the first antibiotically active amiclenomycin peptide in which the essential component occurs N-terminally. No degradation was observed for the antibiotics **1**, **3**, **4** and **8** when performed in buffer solution according to method A. Obviously, using this procedure enzymatic degradation occurs only on N-terminal protein amino acyl bonds. However, when the pronase P cleavage was performed in water (method B) instead of buffer, total degradation of the tripeptides **4**, **5**, and **6** into their amino acid compounds was observed.

Biological Activities

Amiclenomycin is described[10] as an inhibitor of KAPA-DAPA transamination blocking biotin biosynthesis[11]. The di- and tripeptides **1**–**6** particularly inhibit gram-negative bacteria (Table 1). However, *Bacillus subtilis* is also inhibited[12]. No toxic effects were observed within 7 days upon oral or subcutane administration to mice (320 mg/kg). In addition there were no effects on lymphocyte proliferation with or without addition of concanavalin A. Finally it is noteworthy that the free amino acid Acm inhibits *Escherichia coli* in the plate inhibition test, and this action is inhibited by biotin. However, *Okami* et al.[1] reported an activity of Acm *specifically* against mycobacteria. The preparation and spectroscopic identification of enzymatically and chemically modified amiclenomycin peptides will be reported elsewhere[13].

The authors are grateful for the financial support by the *Deutsche Forschungsgemeinschaft* (SFB 76). We thank the MS and NMR departments of the Chemisches Institut der Universität Tübingen: *K. Kolshorn, R. Brettauer, K. P. Voges, H. Bartholomä,* and *G. Nicholson.* We acknowledge also the help of Prof. Dr. *A. Prox, M. Munari* and *G. Farag.* The assistance of *H. B. Sick* during the tedious isolation period is gratefully acknowledged. Furthermore, we thank Prof. Dr. *W. Bessler* for performing the mitogenicity test on lymphocytes.

Table 1. Antibiotic activities of the antimetabolites **1**–**6** [minimal inhibition concentration (MIC) in μg/ml]

Microorganism	Antibiotic					
	1	**2**	**3**	**4**	**5**	**6**
Escherichia coli ATCC 11775 TEM	0.125	0.03	0.06	0.06	0.5	0.01
Escherichia coli 11775	0.25	0.007	0.125	0.06	0.125	0.01
Escherichia coli BC 29 β	0.125	0.03	0.03	0.06	0.25	0.08
Pseudomonas aeruginosa BC 19	> 64	> 64	> 64	64	16	10.5
Pseudomonas aeruginosa Hbg	> 64	> 64	> 64	> 64	> 64	21.0
Klebsiella pneumoniae ATCC 10031	0.06	0.007	0.06	0.25	0.125	0.16
Klebsiella pneumoniae 1082 E	8	0.5	1	0.5	0.5	0.33
Klebsiella pneumoniae BC 6	8	16	1	2	0.5	2.62
Serratia marcescens ATCC 13880	4	1	0.5	0.5	2	1.3
Serratia marcescens BC 8	0.06	0.06	0.06	0.06	0.06	0.04
Enterobacter cloacae ATCC 13047	4	4	8	4	2	1.3
Enterobacter cloacae P 99	0.015	0.03	0.125	0.06	0.125	0.16
Citrobacter freundii ATCC 11601	0.015	0.015	0.015	0.125	0.06	0.04
Proteus inconstans ATCC 25825	0.25	0.007	0.06	0.125	1	0.16
Acinetobacter calcoaceticus ATCC 11959	64	> 64	64	32	16	10.5
Acinetobacter calcoaceticus ATCC 15473	64	> 64	64	32	32	21.0

Experimental

Abbreviations: Acm = amiclenomycin; CD = circular dichroism; CCD = counter-current distribution; Dns = dansyl; FD-MS = field-desorption mass spectrometry; GC = gas chromatography; HPhe = L-homophenylalanine; HPLC = high-performance liquid chromatography; MeIle = *N*-methyl-L-isoleucine; MeVal = *N*-methyl-L-valine; Me = methyl; NMR = nuclear magnetic resonance; nPr = *n*-propyl; Pfp = pentafluoropropionyl; Tfa = trifluoroacetyl; TLC = thin-layer chromatography. Amino acids, peptides and derivatives are abbreviated according to the IUPAC-IUB Commission on Biochemical Nomenclature [see Eur. J. Biochem. **138,** 9 (1984)].

Chemicals: All solvents used for chromatography and CCD were dried, fractionated and degassed prior to use. All reagents and solvents of p.a. quality were from Merck (Darmstadt). DL-Homophenylalanine[14], *N*-methyl-DL-isoleucine[15] were prepared according to the literature, and *N*-methyl-L-valine was purchased from Serva. These amino acids were characterized for comparison by ^{13}C NMR, GC-MS, GC on chiral phase, and as Dns derivatives. – *Thin-layer chromatography:* R_F values of TLC were determined in solvent saturated chambers of Camag (Muttenz). Plates with Kieselgel 60 F_{254} (Merck No 5714); solvent systems: A = chloroform/methanol/water/17-% ammonia (65:35:4:3); B = 1-butanol/acetic acid/water (2:1:1); C = formic acid/water (1:24); D = acetic acid/benzene (1:4); detection with ninhydrin, chlorine/*N,N,N′,N′*-tetramethyl-4,4′-methylenebis(benzenamine) (TDM)[16], and iodine. – Sharp *melting points* (all above 200°C) were not obtained due to partial decompositon. – *Amino acid analyses* were performed on the Biotronik Chromatographic System LC 6000 E using total hydrolysates of the antibiotics (6 N HCl, 110°C, 24 h). L-Homophenylalanine was eluted between L-lysine and L-arginine and determined quantitatively by a standard mixture. Experiments to determine the native antibiotics *via* amino acid analyzer were only partially successfull, due to thermal decomposition (NH_3 elimination) in the heater coil. However, conversion of the antibiotics to the HPhe analogs prior to amino acid analyses gave quantitative results. This method can be recommended also for quantitative determination for the antibiotics **2** and **5**–**7** from culture media. – *Gas chromatography:* Pfp/nPr derivatives were obtained by esterification of the total hydrolysate (1 mg) with 2 N HCl/1-propanol (500 µl) at 100°C for 1 h followed by acylation with pentafluoropropionic anhydride (500 µl) at 150°C for 10 min[17]. Urethane derivatives (for *N*-methylamino acids) were prepared by heating the total hydrolysate (1 mg) with *tert*-butyl isocyanate (200 µl) in dichloromethane (200 µl) at 100°C for 15 min[8]. Determination of the configuration of the amino acid derivatives was carried out on Duran glass capillaries (20 m) coated with Chirasil-Val[7] on a Fraktovap 2150 of Carlo Erba (Hofheim). Temperature program: 80°C/3 min, 80 to 200°C/4 min; hydrogen Pi = 0.4 kg/cm^2, chart 1 cm/min. – *Gas chromatography-mass spectrometry:* Gas chromatograph Fractovap 2900 (Carlo Erba, Hofheim) and mass spectrometer MAT 112 S (Varian, Bremen): samples were esterified with diazomethane and trifluoroacetylated as described below. – *Dansyl Edman degradation* was performed according to *Hartley*[18] as described in ref.[9]. We used the solvent systems C (1st dimension) and D (2nd dimension) and polyamide sheets[19] of Macherey-Nagel & Co (Düren). – *Gel chromatography:* (a) Sephadex G-15 (40–120 µ) and G-25 fine (20–80 µ) (Pharmacia), columns 5 × 100 cm; bed volume 1.6 l; flux rate 60 ml/h, 0.02 N acetic acid (CFG Duramat pump); sample size 5 g; (b) Sephadex LH-20 fine (25–100 µ) methanol/water (7:3); sample size 2 g. Detection of the fractions by TLC, plate diffusion test, and bioautography. – *High-performance liquid chromatography:* Chromatograph (Waters, Königstein) consisting of 2 pumps M 6000 A, programmer M 660, UV detector M 440 (254 and 280 mm) and Chromatograph SP 8000 B (Spectra-Physics, Darmstadt) with spectrophoto-

metric detector 770. Analytical HPLC columns: (a) μ-Bondapak NH_2 (3.9 × 300 mm) of Waters (Königstein) with precolumn Hyperchrome PC (4.6 × 40 mm) of Bischoff (Leonberg) or LiChrosorb NH_2 (5 μ) (Merck) and (b) Hyperchrome column (NC 4.6 × 250 mm) filled with LiChrosorb NH_2 (5 μ) again with precolumn. Preparative HPLC columns: Hyperchrome Präp 2025 (20.5 × 250 mm) (Bischoff) and Vertex (16 × 250 mm) (Knauer), both with LiChrosorb NH_2 (7 μ) (Merck): sample size 10–100 mg. – *Counter-current distribution according to Craig*[20]: apparatus and solvent systems, see Scheme 1. – *^1H NMR spectra* were taken with a WH-90 (90 MHz) and a WM 400 (400.13 MHz) spectrometers of Bruker (Karlsruhe); *^{13}C NMR spectra* were recorded at 22.628 MHz and 100.62 MHz; samples 5–50 mg/0.5 ml D_2O; temperature 30°C. – *Field-desorption* (FD) *mass spectra* were taken with a MAT 711 of Varian (Bremen) coupled with the data system SS 200; temperature of ion source 60°C. – *IR spectra:* c = 2 mg antibiotic/0.5 g KBr; IR spectrometer IFS 114 C of Bruker (Karlsruhe). – *CD spectra:* c = 5 mg antibiotic/2 ml water; d = 0.01 and 0.05 cm; dichrograph CD 185 of Roussel-Jouan (Paris).

Selective conversion of L-*amiclenomycin* (**7**) *to* L-*homophenylalanine:* The finding of increasing amounts of L-homophenylalanine by GC-MS in the total hydrolysates of the fractions of increasing activity was the first hint for the structure of the antimetabolites. A sample of the antibiotic (10 mg) was heated in water (1 ml) at 100°C for 90 min, acidified at 0°C with 1 N HCl and lyophilized. The ammonia content was determined quantitatively. – *Trifluoroacetylated peptide methyl esters:* Gaseous diazomethane (from 10 mg of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) was introduced at 0°C into a methanolic solution (250 μl) of the peptide (1 mg) in a Reacti-Vial (Macherey-Nagel & Co, Düren). After 30 min at room temp. the solvents were removed, and 500 μl of trifluoroacetic anhydride/dichloromethane (1:5) was added. After 10 min at 50°C the solvents were removed in a stream of nitrogen, and the residue was dissolved in dichloromethane for GC-MS.

Characterization of the antimetabolites **1**–**6**. – TLC: Among several TLC systems used the system A was found to be the most indicative: R_F (A) = 0.29 (**1**), 0.23 (**2**),*0.20* (**3**),0.16 (**4**), 0.13 (**5**), 0.10 (**6**); R_F (B) = 0.35 (**1**), 0.48 (**2**), 0.29 (**3**), 0.21 (**4**), 0.25 (**5**), 0.22 (**6**). – The spectrometric data of **1**–**6** are summarized in Tables 2–7.

Characterization of L-*Melle-*L-*Acm-*L-*Glu* (**10**)*: This tripeptide antibiotic was isolated from the *S. venezuelae* strain Tü 2605. – R_F (A) = 0.09, R_F (B) = 0.33. – ^1H NMR (D_2O, 400.13 MHz): δ = 0.95 (t; 3H, Melle γ-H), 0.98 (d; 3H, Melle γ-H), 1.23 (m; 1H, Melle γ-H_A), 1.57 (m; 1H, Melle γ-H_B), 1.66 (m; 2H, Acm 4-H), 1.79 (m; 1H, Acm 3-H_A), 1.92 (m; 1H, Acm 3-H_B and Glu β-H_A), 2.08 (m; 1H, Melle β-H and Glu β-H_B), 2.30 (t; 2H, Glu γ-H), 2.71 (s; 3H, Melle NCH_3), 2.95 (s, broad; 1H, Acm 5-H), 3.81 (d; 1H, Melle α-H), 4.18 (m; 1H, Acm 2-H), 4.42 (m; 1H, Acm 8-H and Glu α-H), 5.87 (m; 2H, Acm 6-H and 10-H), 6.15 (m; 2H, Acm 7-H and 9-H). – ^{13}C NMR (D_2O, 100.6 MHz): δ = 13.42 (Melle C-δ), 16.1 (Melle C-γ'), 27.5 (Melle C-γ), 30.1 (Glu C-β and Acm C-4), 31.9 (Acm C-3), 34.5 (Glu C-γ), 34.8 (Melle NCH_3), 36.9 (Acm C-5), 38.5 (Melle C-β), 47.4 (Acm C-8), 56.6 (Glu C-α and Acm C-2), 68.5 (Melle C-α), 123.3 (Acm C-6), 123.4 (Acm C-10), 137.7 (Acm C-7), 137.75 (Acm C-9), 170.0 (Melle C=O), 174.6 (Glu C=O), 180.1 (Acm C=O), 181.9 (Glu C-δ). – FD-MS: m/e = 474 (10%, $[M - 1 + Na]^+$), 453 (4%, $[M + 1]^+$), 436 (100%, $[M + 1]^+ - NH_3$.

*) Supplemented on correction (Nov. 7, 1984); details will be reported elsewhere[13].

Table 2. ^{13}C NMR data (δ values) of the peptide antibiotics **1**–**6** in D_2O (pH = 7)

Antibiotic	Amiclenomycin carbons								Amino acid carbons							
	α	β	γ	δ	ε,ε′	ζ,ζ′	η	CO		α	β	γ	γ′	δ	CO	NCH_3
MeIle-Acm (**1**)	57.8	32.1	31.2	37.3	123.5, 123.5	138.0, 138.0	47.6	180.6	MeIle	70.5	39.7	27.7	17.3	13.3	174.9	35.8
Ile-Acm (**2**)	58.5	32.3	31.2	37.4	123.1	138.5	47.8	180.5	Ile	60.6	39.2	26.8	16.9	13.3	171.2	
MeVal-Acm (**3**)	58.4	32.1	30.6	37.0	123.1	137.9, 137.9	47.4	180.3	MeVal	69.7	32.4	20.3	19.8		169.6	34.7
MeIle-Acm-Gln (**4**)	57.5	32.3	30.6	37.3	123.3, 123.4	138.2, 138.3	47.7	180.3	MeIle Gln	68.9, 57.5	39.0, 30.5	27.7, 34.4	16.4	13.4, 181.1	170.3, 174.5	35.1
Ile-Acm-Gln (**5**)	57.1	32.0	30.4	37.0	123.7	137.6	47.3	180.0	Ile Gln	61.5, 56.2	40.8, 30.4	26.6, 34.0	17.4	13.1, 180.9	 175.2	
Val-Acm-Gln (**6**)	56.6	31.3	30.1	36.9	123.3	137.6	47.2	180.7	Val Gln	60.5, 56.6	30.1, 30.1	20.1, 34.0	19.6	 180.7	171.9, 175.0	

Table 3. ^1H NMR data (δ values) of the peptide antibiotics **1**–**6** in D_2O (pH = 7)

Antibiotic	Amiclenomycin							Amino acids						
	α	β	γ	δ	ε,ε′	ζ,ζ′	η		α	β	γ	γ′	δ	NCH_3
MeIle-Acm (**1**)	4.22	1.73, 1.83	1.59	2.93	5.84	6.12	4.40	MeIle	3.80	2.02	1.30, 1.59	1.00	0.95	2.68
Ile-Acm (**2**)	4.17	1.72, 1.82	1.59	2.92	5.84	6.13	4.41	Ile	3.89	2.00	1.26, 1.59	1.03	0.94	
MeVal-Acm (**3**)	4.22	1.72	1.59	2.92	5.84	6.12	4.40	MeVal	3.73	2.25	1.08	1.02		2.69
MeIle-Acm-Gln (**4**)	4.15	1.75, 1.84	1.62	2.90	5.83	6.12	4.38	MeIle	3.78	2.08	1.22, 1.54	0.95	0.92	2.68
								Gln	4.43	1.95	2.30			
Ile-Acm-Gln (**5**)	4.15	1.74	1.62	2.90	5.83	6.12	4.37	Ile	3.88	2.08	1.21, 1.48	0.98	0.91	
								Gln	4.37	1.93	2.30			
Val-Acm-Gln (**6**)	4.18	1.78, 1.85	1.65	2.92	5.85	6.15	4.39	Val Gln	3.84 4.39	2.23 1.95 2.10	1.02 2.33			

Table 4. Results of $\{^1H/^1H\}$ and $\{^1H/^{13}C\}$ double-resonance experiments with Melle-Acm (1)
[SFD = single-frequency decoupling]

	SFD [ppm]	Collapse of multipletts [ppm]							
		6.12	5.84	4.40	4.22	2.93	1.83	1.73	1.59
Acm ζ-,ζ'-H	6.12	↯	+	+		+			
Acm ε-,ε'-H	5.84	+	↯	+		+			
Acm η-H	4.40	+		↯		+			
Acm α-H	4.22				↯			+	(+)
Acm δ-H	2.93	+	+	+		↯			+
Acm β-H	1.83				+		↯	(+)	+
Acm β-H	1.73				+			↯	+
Acm γ-H	1.59					+	(+)	(+)	↯
		3.80	2.02	1.59	1.30	1.00	0.95		
Melle α-H	3.80	↯							
Melle β-H	2.02	+	↯		+	+			
Melle γ-H	1.59			↯	+		+		
Melle γ-H	1.30			+	↯		+		
Acm ζ-,ζ'-H	6.2	Acm C-7, C-9			138	d → s			
Acm ε-,ε'-H	5.8	Acm C-6, C-10			123	d → s			
Melle α-H	3.8	Melle C-2			70	d → s			
Acm δ-H	2.9	Acm C-5			37.5	d → s			

Table 5. Assignments of the IR absorption bands ($\tilde{\nu}$, cm^{-1}) of the 1,4-cyclohexadienyl ring system in the antibiotics **1**–**6** in comparison to the unsubstituted 1,4-cyclohexadiene[21]

	Chd[a]	Antibiotics					
		1	**2**	**3**	**4**	**5**	**6**
Ring breathing	854	860	860	860	860	855	865
CH stretching	3032	3040	3040	3040	3070	3030	3040
Ring deformation	530	520	530	540	550	530	535
Ring-C=C stretching	1639	1650	1640	1650	1660	1660	1660
In plane MC=CH bending	1197	1195	1190	1190	1190	1185	
Out-of-plane MC=CH bending	985	990	1000	1000	990	985	985
stretching	1377	1360	1360	1360	1360	1350	1350

[a] Chd = 1,4-cyclohexadiene.

Fermentation: (a) Complex medium with meat and malt extracts in water (pH = 7.2); (b) minimal medium containing glycerol, glucose, potassium dihydrogen phosphate, ammonium sulfate, magnesium hydrogen phosphate, and trace elements in water (pH = 5.5). Details are described elsewhere[12].

Biological Assays: Agar plates containing Braun Flexner Agar and top agar with *Escherichia coli* 12593/74 at an incubation temp. of 37°C for 15 h were used. Plate diffusion

Table 6. Results of field-desorption mass spectrometry

	$[M-1+Na]^+$	$[M+1]^+$	M^+	$[M+1]^+ - NH_3$	$M^+ - NH_3$
1		324 (100%)		307 (30%)	
2		310 (30%)	309 (5%)	293 (100%)	292 (8%)
3		310 (100%)	309 (25%)	293 (95%)	292 (22%)
4		452 (4%)	451 (2%)	435 (100%)	
5	459 (55%)		437 (8%)	421 (100%)	
6	445 (57%)	424 (30%)		407 (100%)	

Table 7. Circular dichroism data of aqueous solutions of the peptide antibiotics **1**–**6**

Peptide	λ_{max} [nm]	Molar ellipticity $[\theta] \times 10^3$ [deg × cm² × dmol⁻¹]
1	218	−7.5
2	214	−3.5
3	213	−2.6
4, 5	negative ellipticities increasing from λ = 250 nm to about 210 nm	
6	no Cotton effect	

tests were performed using filter rondelles (6 mm; Macherey-Nagel & Co) with 20 µl of the antibiotic solutions.

Bioautography: Developed and dried TLC plates were covered with self-adhesive plastic sheeting, detached and divided into stripes (1 mm). Each stripe was tested separately on agar test plates by incubation at 37°C for 15 h.

Enzymatic cleavage [22] *with Pronase P. – Method A:* Pronase P from *Streptomyces griseus* (Serva, Heidelberg) (0.5 mg) was dissolved in 1 ml of 0.01 M Na_2HPO_4 buffer solution [3.58 g $Na_2HPO_4 \cdot 12\ H_2O$ in 1 l of NaCl solution (0.9%), adjusted to pH = 7.0 with 1 N HCl]. Immediately after dissolution the enzyme solution (10 µl) was pipetted to the antibiotic solution (100 µl; c = 5 mg/ml in 0.01 M Na_2HPO_4 buffer). After 8 h at 37°C the solution was analyzed by TLC and amino acid analyzer.

Method B: Pronase P (5 mg) was dissolved in 1 ml of H_2O and added to the antibiotic solution (5 ml; c = 20 mg/ml H_2O). After 20 h at 37°C the solution was analyzed as described above.

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