CLAVAMYCINS, NEW CLAVAM ANTIBIOTICS FROM TWO VARIANTS OF *STREPTOMYCES HYGROSCOPICUS*

II. ISOLATION AND STRUCTURES OF CLAVAMYCINS A, B AND C FROM *STREPTOMYCES HYGROSCOPICUS* NRRL 15846, AND OF CLAVAMYCINS D, E AND F FROM *STREPTOMYCES HYGROSCOPICUS* NRRL 15879

HANS ULRICH NAEGELI*, HANS-RUDOLF LOOSLI and ALEX NUSSBAUMER

Sandoz Ltd., Preclinical Research, CH-4002 Basel, Switzerland

(Received for publication November 15, 1985)

Clavamycins A, B, C, D, E and F represent new members of the clavam antibiotics group. Their purification was achieved by a variety of preparative chromatographic methods, essentially on reversed phase carrier. The structures were deduced from spectroscopic properties, especially from extensive NMR studies.

In the preceding report[1], taxonomy of the producing strains, fermentation and biological activities of new clavam antibiotics from two *Streptomyces* strains NRRL 15846 and NRRL 15879 are presented. In this paper we describe details of isolation, physico-chemical characterization and the structures of six new clavams, clavamycins A~F.

The non-extractable clavamycins A~F were highly unstable during isolation procedures. Therefore all purification steps, except for preparative HPLC, were run at 4°C, and chromatographic fractions were stored at −90°C. Concentration steps *in vacuo* were not completed to dryness, otherwise an unusual loss of bioactivity resulted.

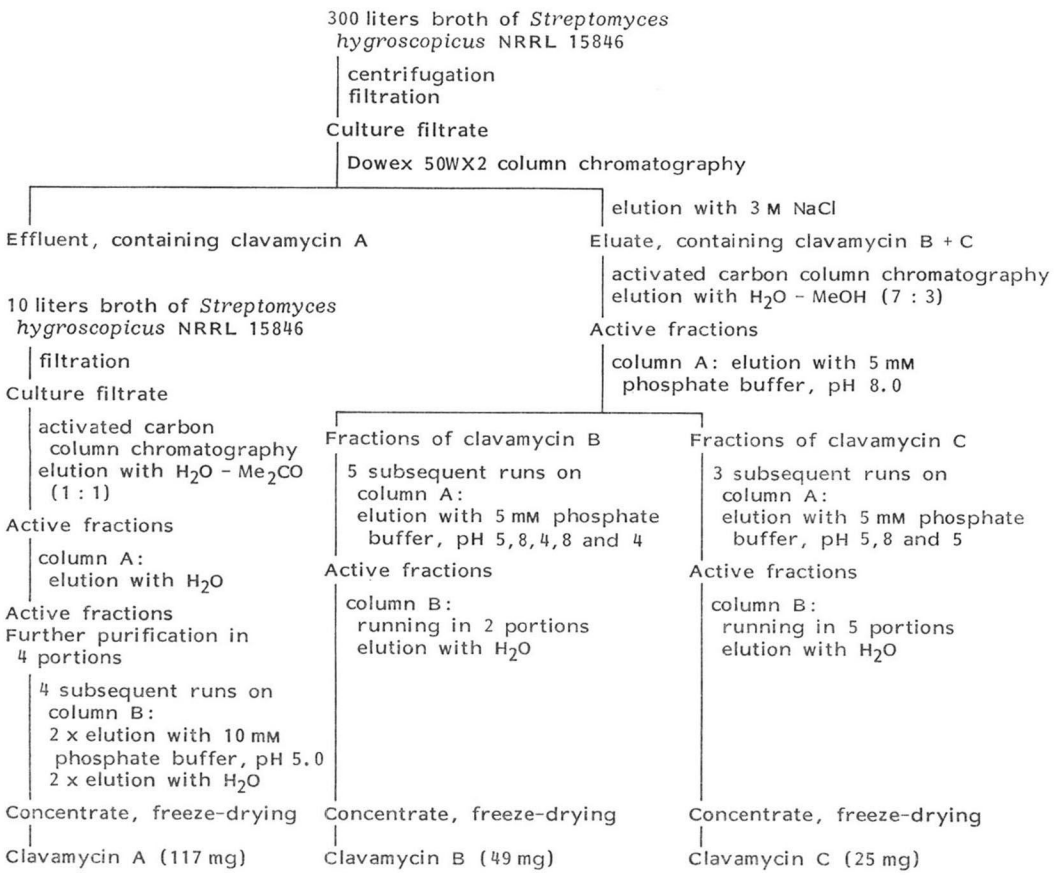
In addition to the non-extractable clavamycins A~C from strain *Streptomyces hygroscopicus* NRRL 15846, an antifungal activity could be isolated by dichloromethane extraction, which was identified as the known antibiotic hydroxymethylclavam[2].

Isolation of Clavamycins A, B and C from *S. hygroscopicus* NRRL 15846

Clavamycin A

Ten-liter culture broth of *S. hygroscopicus* NRRL 15846[1] was adjusted with 4 N H_2SO_4 to pH 7, cooled to 4°C and filtered through Celite 545 (see Fig. 1). The filtrate was applied to a 1-liter column of activated carbon (Wako). After washing with 1 liter of water, the column was eluted with 2×500 ml portions of water - acetone (9: 1), then with 6×500 ml portions of water - acetone (1 : 1). Active fractions (2 liters) were concentrated *in vacuo* at 10°C and further chromatographed on a 5×40 cm medium pressure column of LiChroprep RP 18 (40~64 μm), with water elution. The resulting active fractions were concentrated *in vacuo* at 10°C. The concentrate was divided into 4 equal portions and each purified twice on a preparative 16×250 mm HPLC column of LiChrosorb RP 18 (10 μm), with 10 mM phosphate buffer (pH 5) elution. Active fractions were again chromatographed twice on the same

Fig. 1. Purification scheme of clavamycins A, B and C.
Column A: 5×40 cm medium pressure column of LiChroprep RP 18 (40~64 μm). Column B: 16×250 mm HPLC column of LiChrosorb RP 18 (10 μm).



HPLC column as above, eluting with deionized water. Fractions giving a single peak of clavamycin A by analytical HPLC were concentrated and freeze-dried, yielding a total of 117 mg clavamycin A from all 4 portions as a white amorphous powder, mp >190°C (dec), $[\alpha]_D^{25}$ −86.8° (*c* 0.66, H_2O).

Clavamycins B and C

A 300-liter fermentation broth of *S. hygroscopicus* NRRL 15846[1] was adjusted to pH 7 with 4 N H_2SO_4, cooled to 4°C and clarified by centrifugation and then by filtration through Celite 545 (see Fig. 1). The filtrate was applied to a 20×36 cm column (11 liters) of Dowex 50WX2 (Na^+ type, 50~100 mesh). Clavamycins B and C were retained, whereas clavamycin A was found in the effluent. The latter could be further adsorbed on activated carbon and purified as described in the preceding section. After washing with 17 liters of water, the Dowex column was eluted with 6×5-liter portions of 3 M NaCl. Four active fractions were collected and applied to a 1-liter column of activated carbon (Wako) to remove the NaCl. The column was washed with 1 liter of water and then developed with water - methanol (7: 3). The eluate (4 liters) was concentrated *in vacuo* at 10°C. Final purification of clavamycins B and C was attained by medium pressure chromatography and preparative HPLC in several subsequent runs on reversed phase material according to Fig. 1, whereas fractions exhibiting

the major activity were collected. Excellent selectivity was achieved only by varying the pH of the eluting buffers. Separation monitoring was performed on analytical HPLC with a HP 1040 diode array detector.

Isolation of Clavamycins D, E and F from *S. hygroscopicus* NRRL 15879

A 200-liter culture broth of *S. hygroscopicus* NRRL 15879[1] was adjusted to pH 7 with 4 N H_2SO_4, cooled to 4°C and clarified by centrifugation and then by filtration through Celite 545 (see Fig. 2). The filtrate was percolated through a 20×48 cm column (15 liters) of activated carbon (Wako). After washing with 15 liters of water, the column was eluted with 3×5-liter portions of water - methanol (9: 1), then with 6×5-liter portions of water - methanol (1: 1). Fractions 3~6, containing the major antibiotic activity, were concentrated *in vacuo* at 10°C to 2 liters, then adjusted with phosphate buffer to pH 8.0 and to a final buffer concentration of 0.1 M. This concentrate was applied to a 12×35 cm medium pressure column (4 liters) of LiChroprep RP 18 (40~64 μm), then eluted in 500-ml fractions with a linear decreasing gradient between 0.1 M phosphate buffer (pH 8.0) and water. This chromatography gave a separation of clavamycin D from E and F: The first 4 active fractions contained the major quantity of clavamycin D, whereas the subsequent 3 fractions contained clavamycins E and F (monitored by analytical HPLC).

Fractions of clavamycin D (2 liters) were collected and concentrated *in vacuo* at 10°C, adjusted to pH 7.2 and chromatographed twice on a 5×40 cm medium pressure column of LiChroprep RP 18 (40~64 μm), eluting with 10 mM phosphate buffer, pH 7.2. Active fractions were rechromatographed on

Fig. 2. Purification scheme of clavamycins D, E and F.

Column A: 12×35 cm medium pressure column of LiChroprep RP 18 (40~64 μm). Column B: 5×40 cm medium pressure column of LiChroprep RP 18 (40~64 μm). Column C: 16×250 mm HPLC column of LiChrosorb RP 18 (10 μm).

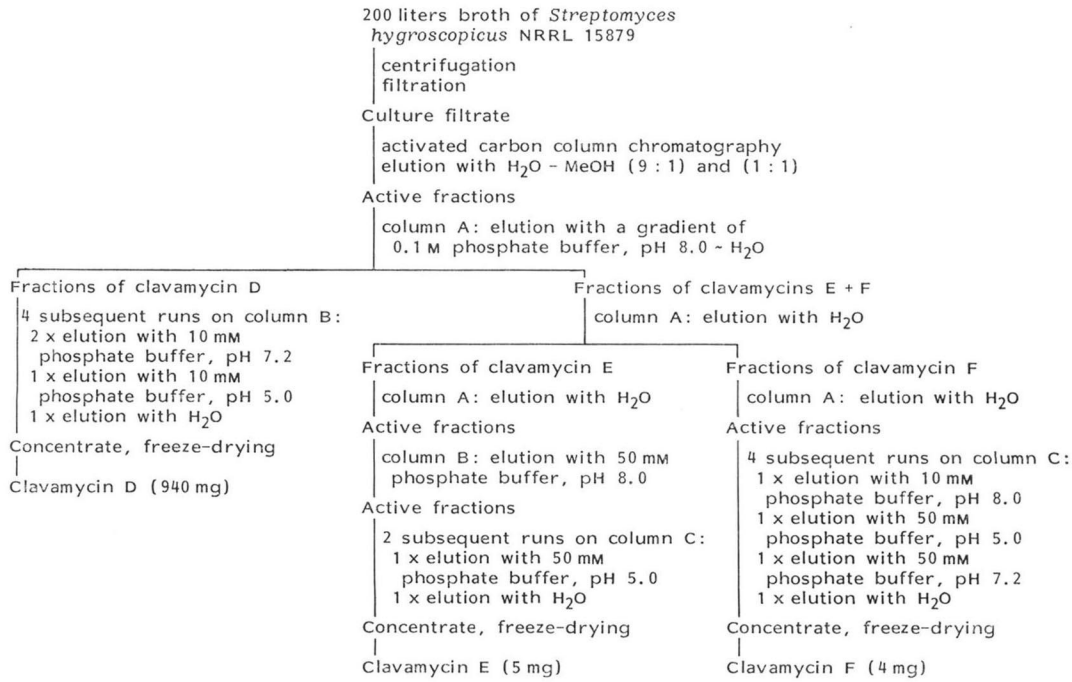


Table 1. HPLC and TLC data of clavamycins A, B and C.

Compound	HPLC system A retention times (minutes)			TLC Rf values
	pH 5	pH 7.2	pH 8	
Clavamycin A	6.3	9.1	9.2	0.52
〃 B	3.0	3.5	4.0	0.14
〃 C	3.7	3.1	3.0	0.1

HPLC system A: LiChrosorb RP 18 (4.6×250 mm), 10 mM phosphate buffer, flow rate: 2 ml/minute. UV detection at 220 nm.
TLC: Cellulose Polygram Cell 300, propanol - H_2O (7: 3). Detection by bioassay against *Candida albicans*.

Table 2. HPLC and TLC data of clavamycins D, E and F.

Compound	HPLC system B retention times (minutes)		TLC Rf values
	pH 5	pH 7.2	
Clavamycin D	2.9	5.8	0.68
〃 E	2.7	2.1	0.43
〃 F	5.8	4.7	0.51

HPLC system B: Chrompack Spher C 18 (3×200 mm), 50 mM phosphate buffer, flow rate: 1 ml/minute. UV detection at 220 nm.
TLC: See Table 1.

the same column, eluting with 10 mM phosphate buffer, pH 5.0. HPLC-pure fractions of clavamycin D were pooled and desalted on the 5×40 cm RP 18 column as above with deionized water. Concentration and freeze-drying gave 940 mg clavamycin D as a white powder, mp >210°C (dec), $[\alpha]_D^{25}$ −41.3° (*c* 0.76, H_2O).

Fractions of clavamycins E and F were pooled, concentrated *in vacuo* at 10°C and again chromatographed on the 12×35 cm column of RP 18 carrier, with water eluting. The first active fractions contained clavamycin E and the subsequent clavamycin F. Final purification of both antibiotics was attained in several runs on reversed phase carrier by varying the pH and the ionic strength of the eluting buffer, according to Fig. 2.

Physico-chemical Properties

Clavamycins A~F were isolated as white amorphous powders by freeze-drying, whereas clavamycin D could be crystallized from cold methanol. They were soluble in water and DMSO, dissolved moderately in methanol and were insoluble in chloroform, ethyl acetate and hexane. The stability of the purified clavams in aqueous buffer solutions was surprisingly better than was expected due to our previous isolation experience. In 10 mM phosphate buffer at a pH of 5~8 they could be stored at 4°C for some days without significant loss of activity. The lyophilized powders were stable at −90°C for 6 months. The UV spectra of the clavams (10 mM phosphate buffer, pH 7) exhibited only end absorption, whereas the IR spectra (KBr disc) indicated the dominant absorption at 1780~1765 cm^{-1} of the β-lactam carbonyl group. Chromatographic data on TLC and HPLC are listed in Tables 1 and 2.

Structures of Clavamycins A, B and C from *S. hygroscopicus* NRRL 15846

Clavamycin A (1)

The molecular weight of **1** is 414, determined by FAB-MS (*m/z* 415, M+H). Microtitration gives evidence for COO^- (IR 1610 cm^{-1}, ^{13}C NMR 173.21 ppm) and for NH_3^+ (IR 2950 cm^{-1}). The ^{13}C

Fig. 3. Structures of clavamycins A, B and C.
For numbering of the positions see text.

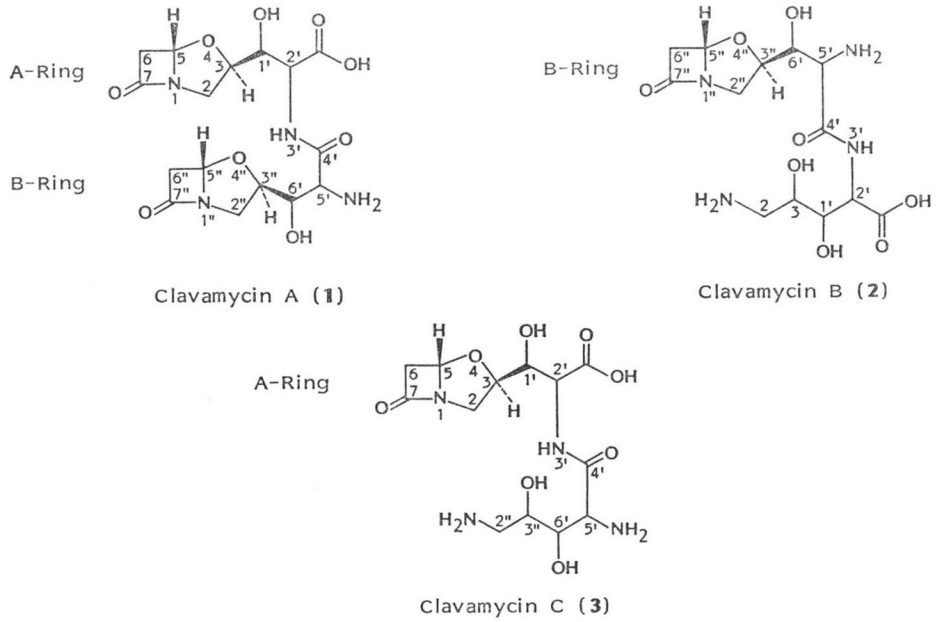
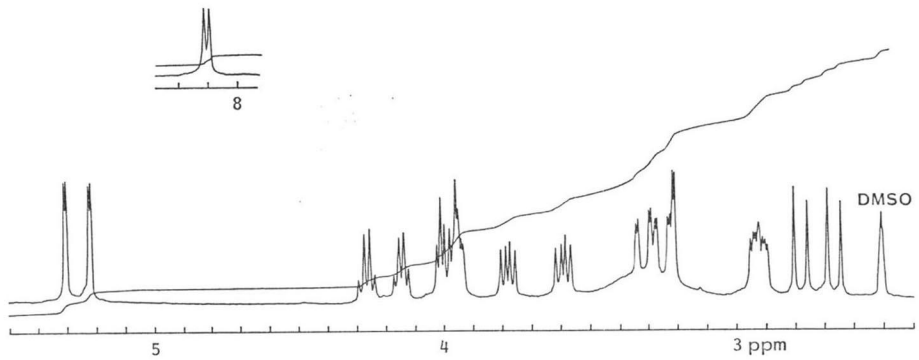


Fig. 4. ^{1}H NMR spectrum of clavamycin A (**1**) in DMSO-d_6.



and ^{1}H NMR spectra (Fig. 4) show a duplication of several resonance signals with chemical shift data and coupling constants indicating the presence of two similar clavam substructures, linked by an amide bond. The corresponding amide signal is observed in the ^{1}H NMR at 8.09 ppm and in the ^{13}C NMR at 172.59 ppm (DMSO-d_6). From data of NOE spectroscopy, double resonance experiments and proton-carbon correlated spectroscopy (optimized on 135 Hz and 5 Hz coupling constants), structure **1** results. All assignments, based on NMR experiments, are presented in Table 3.

Clavamycins B (**2**) and C (**3**)

Metabolites **2** and **3** both have the same molecular weight of 362, determined by FAB-MS (*m/z* 363, M+H), and the characteristic IR and NMR data of a clavam. The NMR data, however, show no duplication of resonance signals as is seen for structure **1**, but coupling constants and chemical shift data indicate a very close relationship of **2** and **3** with **1** (Table 3). Extensive NMR experiments, in-

Table 3. Assignments of the ^1H NMR and ^{13}C NMR spectra of **1**, **2** and **3** in ppm (δ).

Position	**1** in DMSO-d_6		**2** in DMSO-d_6		**2** in D_2O		**3** in D_2O	
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
2	2.96, 3.65	46.53	2.80, 2.95	41.83	3.06, 3.39	43.32	3.08, 3.97	50.32
3	4.18	82.59	3.80	68.40	3.68	68.81	4.29	84.60
5	5.25	83.42	—	—	—	—	5.46	87.37
6	2.69, 3.26	44.35	—	—	—	—	2.91, 3.38	47.20
7	—	178.05	—	—	—	—	—	*
1′	4.06	71.05	3.80	72.57	4.08	74.42	4.29	74.71
2′	4.10	55.41	4.08	55.52	4.61	57.10	4.53	58.76
2′ -COO^-	—	173.21	—	173.97	—	177.00	—	*
3′	8.09	—	8.32	—	—	—	—	—
4′	—	172.59	—	172.52	—	175.15	—	*
5′	3.28	56.52	3.28	56.46	3.86	57.60	3.73	59.14
6′	3.97	71.63	3.98	71.58	4.13	73.24	3.92	76.24
2″	2.95, 3.80	47.53	2.95, 3.80	47.67	3.08, 4.09	49.37	3.04, 3.38	45.16
3″	4.29	81.36	4.28	81.27	4.46	82.23	3.92	70.39
5″	5.31	83.74	5.31	83.75	5.49	86.10	—	—
6″	2.79, 3.31	44.61	2.79, 3.31	44.65	2.95, 3.39	45.71	—	—
7″	—	178.05	—	178.04	—	182.83	—	—

* Signals are not detected due to too low concentration of metabolite.

cluding NOE-, double resonance- and H-C correlated spectroscopy (optimized on 135 Hz and 5 Hz coupling constants) reveal for clavamycin B structure **2** and for clavamycin C structure **3**. The latter is tentative, because the quality of the H-C correlated spectra was unsatisfactory due to a decomposition of the clavam during the spectroscopic run.

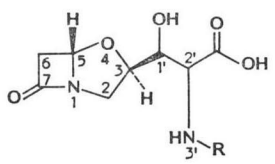
The numbering of the positions of **1**, **2** and **3** (Fig. 3 and Table 3) is chosen to illustrate the close structural relationship. Clavam **2** represents clavam **1** lacking the β-lactam ring A and **3** represents **1** lacking ring B. There is no evidence whether **2** or **3** are precursors or artifacts of **1**.

Structures of Clavamycins D, E and F from *S. hygroscopicus* NRRL 15879

Clavamycin D (4)

The molecular formula $C_{13}H_{21}N_3O_6$ of **4** is set up by elemental analysis, ^{13}C NMR data and FAB-MS, showing a peak at m/z 316 (M+H). The presence of a clavam is obvious by IR and ^1H NMR data (Fig. 6). Acidic hydrolysis of **4** (6 N HCl, 16 hours, 115°C) gives L-valine, identified by GC on a chiral carrier. Microtitration gives evidence for a COO^- and a NH_3^+ group. These findings together with the assignments based on extensive ^1H and ^{13}C NMR experiments (Table 4) reveal structure **4**.

Fig. 5. Structures of clavamycins D, E and F.



Clavamycin D (**4**) R=L-Valyl
Clavamycin E (**5**) R=L-Alanyl
Clavamycin F (**6**) R=N^δ-Acetyl-ornithyl
7 R=H

Structure **4** is confirmed by preparation of the corresponding *N*-acetyl methyl ester **8** (Fig. 7) and by the enzymatic cleavage of **4** with amino-peptidase, furnishing L-valine and the clavam **7** (Fig. 5). Biological activity of the latter is described in the preceding paper[1].

Clavamycins E (5) and F (6)

5 and **6** differ from **4** only in the amino acid side chain. The molecular weight of **5** is 287

Fig. 6. ^{1}H NMR spectrum of clavamycin D (**4**) in DMSO-d_6.

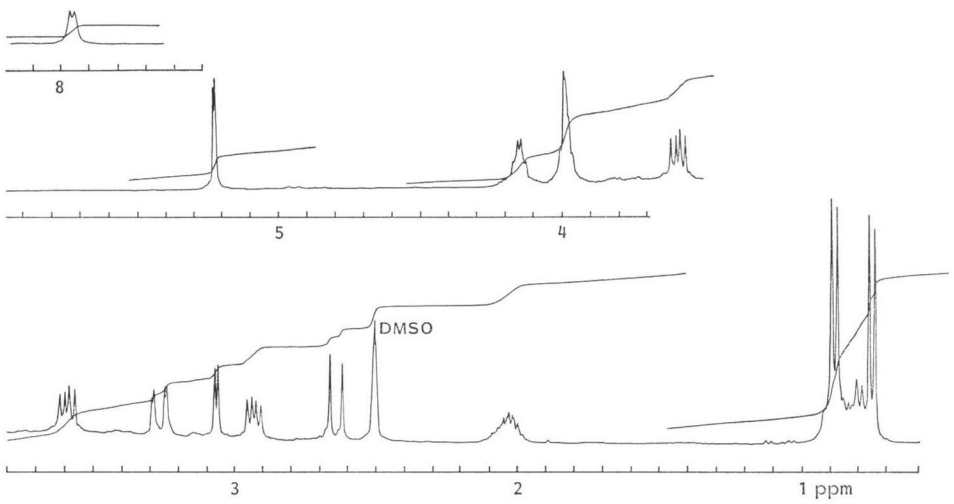


Table 4. Assignments of the ^{1}H NMR and ^{13}C NMR spectra of **4**, **5** and **6** in ppm (δ).

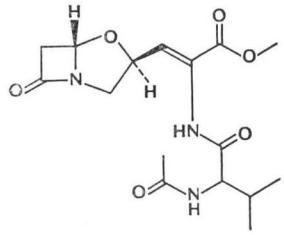
Position	**4** in DMSO-d_6		**5** in DMSO-d_6	**5** in D_2O	**6** in DMSO-d_6	
	^{1}H	^{13}C	^{1}H	^{1}H	^{1}H	^{13}C
2	2.94, 3.61	46.19	2.96, 3.63	3.10, 3.98	2.96, 3.66	45.95
3	4.15	82.04	4.19	4.32	4.18	82.48
5	5.24	83.34	5.25	5.48	5.26	83.35
6	2.65, 3.27	44.50	2.69, 3.28	2.92, 3.41	2.70, 3.29	44.40
7	—	178.10	—	—	—	178.14
1′	4.00	70.90	4.04	4.27	4.04	70.72
2′	4.04	54.42	4.11	4.50	4.18	54.45*
2′ –COO^-	—	171.84	—	—	—	172.31
3′	7.98	—	8.05	—	8.08	—
Val-CO	—	172.59	—	—	—	—
Val-α	3.16	59.24	—	—	—	—
Val-β	2.04	30.68	—	—	—	—
Val-γ, γ'	0.78, 0.90	16.54, 19.25	—	—	—	—
Ala-α	—	—	3.63	3.87	—	—
Ala-β	—	—	1.25	1.45	—	—
Orn-CO	—	—	—	—	—	174.21
Orn-α	—	—	—	—	3.55	54.58*
Orn-β	—	—	—	—	1.55, 1.67	32.40
Orn-γ	—	—	—	—	1.48	25.46
Orn-δ	—	—	—	—	3.02	38.42
Orn-δ-NH	—	—	—	—	7.94	—
Orn-δ-NHCOCH_3	—	—	—	—	1.80	22.48
Orn-δ-NHCO	—	—	—	—	—	168.82

* Signals could be interchanged.

(FAB-MS m/z 288, M+H) and acid hydrolysis (6 N HCl, 16 hours, 115°C) gives L-alanine, identified by GC on a chiral carrier. From this finding and from ^{1}H NMR data (Table 4) structure **5** is elucidated for clavamycin E.

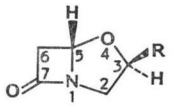
For clavamycin F, having a molecular weight of 372 (FAB-MS m/z 373, M+H), we propose structure **6**. The amino acid side chain, N^δ-acetyl-ornithine, is inferred only by ^{1}H NMR and ^{13}C NMR

Fig. 7.



8

Fig. 8. 3-Substituted 7-oxo-1-aza-4-oxabicyclo-[3.2.0]heptane.



data (Table 4) and not by acidic hydrolysis due to lack of sufficient substance.

Stereochemistry

The relative configuration of the protons in position 3 and 5 in a 3-substituted 7-oxo-1-aza-4-oxabicyclo[3.2.0]heptane derivative (Fig. 8) is established according to the chemical shift difference of the methylene protons in position 2 in the ^{1}H NMR spectra[3,4]. In a *syn* relationship the difference in chemical shift is 0.4~0.5 ppm, and in an *anti* relationship 1~1.4 ppm. Clavamycins A~F show differences of 0.65~0.86 ppm. However the derivatives **7** and **8**, obtained from clavamycin D (**4**), have differences of 1.05 and 1.43 ppm, respectively. Therefore **7**, **8** and also **4** have an *anti* configuration. Due to the very close relationship in ^{1}H NMR data of clavamycins A~F, we propose the *anti* configuration for all of them.

The absolute stereochemistry in position 5 can be determined by CD spectroscopy[4,5]. Derivative **7** shows a negative Cotton effect at 229 nm (θ=52,500), indicating the *S* configuration at position 5. This finding is supported by the antifungal activity of **7**. Antifungal clavams have the *S* configuration at the ring junction position 5, whereas clavams with the *R* configuration inhibit β-lactamases[4]. Because our clavamycins are not β-lactamase inhibitors, but only show antifungal activity, we postulate the *S* configuration at the ring juncture. Thus the stereostructure of clavamycins A~F is 3*R*,5*S* according Fig. 8.

Experimental

NMR spectra were run on a Bruker WH-360 or AN-360 using TMS or 3-(trimethylsilyl)propionic acid sodium salt=0 ppm as an internal standard.

3-(7-Oxo-1-aza-4-oxabicyclo[3.2.0]hept-3-yl)serine (**7**)

To a solution of 10 mg (0.03 mM) **4** in 300 μl 10 mM phosphate buffer (pH 7) was added 100 μl aminopeptidase M (Boehringer, 5 mg/ml). After standing the solution at 37°C for 17 hours, there was no more educt **4** visible by HPLC. The reaction mixture was purified by preparative HPLC on a 16×250 mm column of LiChrosorb RP 18, eluting with 10 mM phosphate buffer, pH 5. Fractions containing **7** were pooled, desalted on LiChrosorb RP 18 with deionized water elution, then freeze-dried, affording 2.2 mg (32%) of **7** as a white powder: FAB-MS *m/z* 217 (M+H); ^{1}H NMR (360 MHz, D_2O) δ 5.50 (1H, d, J=3 Hz, 5-H), 4.43 (1H, q, J=7 Hz, 3-H), 4.26 (1H, dd, J=7 and 3.5 Hz, 2′-H), 4.12 (1H, dd, J=12 and 6.5 Hz, 2-H_a), 3.88 (1H, d, J=3.5 Hz, 2′-H), 3.41 (1H, dd, J=17 and 3 Hz, 6-H_a), 3.07 (1H, dd, J=12 and 6.5 Hz, 2-H_b), 2.95 (1H, d, J=17 Hz, 6-H_b): ^{13}C NMR (90.5 MHz, D_2O) δ 182.46 (C-7), 173.22 (2′-COO), 86.15 (C-5), 82.37 (C-3), 70.87 (C-1′), 57.12 (C-2′), 48.89 (C-2), 45.58 (C-6).

N-Acetyl-valyl-3-(7-oxo-1-aza-4-oxabicyclo[3.2.0]hept-3-yl)dehydroalanine Methyl Ester (**8**)

A mixture of 20 mg (0.06 mM) **4** in 4 ml of acetic anhydride - pyridine (1:1) was stirred at 4°C for 60 hours. The residue obtained after evaporation *in vacuo* was taken up in 4 ml anhydrous methanol and stirred at 20°C for 20 minutes. After dilution with 30 ml water, the solution was extracted with

chloroform and the extract evaporated to dryness *in vacuo*. The residue was chromatographed on silica gel, eluting with dichloromethane - methanol (95: 5), then on a 16×250 mm HPLC column of LiChrosorb RP 18, eluting with water - acetonitrile (4: 1). Fractions containing pure **8** were pooled and freeze-dried, affording 1.2 mg (5%) of a white powder: FAB-MS *m/z* 354 (M+H): UV $\lambda_{max}^{H_2O}$ nm end, 235 (sh): IR (CH_2Cl_2) cm^{-1} 3430, 1785 (s, β-lactam C=O), 1725 (s, ester C=O), 1705 (s, amide C=O), 1675 (s, amide C=O): ^1H NMR (360 MHz, $CDCl_3$) δ 7.82 (1H, s), 6.40 (1H, d), 6.00 (1H, d), 5.41 (1H, d), 4.89 (1H, q), 4.38 (1H, t), 4.28 (1H, dd), 3.82 (3H, s), 3.31 (1H, dd), 2.88 (1H, d), 2.84 (1H, dd), 2.17 (1H, m), 2.06 (3H, s), 1.00 (3H, d), 0.96 (3H, d).

References

1) KING, H. D.; J. LANGHÄRIG & J. J. SANGLIER: Clavamycins, new clavam antibiotics from two variants of *Streptomyces hygroscopicus*. I. Taxonomy of the producing organisms, fermentation, and biological activities. J. Antibiotics 39: 510~515, 1986
2) BROWN, D.; J. R. EVANS & R. A. FLETTON: Structures of three novel β-lactams isolated from *Streptomyces clavuligerus*. J. Chem. Soc. Chem. Commun. 1979: 282~283, 1979
3) BENTLEY, P. H. & E. HUNT: Total synthesis of β-lactamase inhibitors based on the 4-oxa-1-azabicyclo-[3.2.0]heptan-7-one ring system. J. Chem. Soc. Perkin Trans I 1980: 2222~2227, 1980
4) MÜLLER, J.-C.; V. TOOME, D. L. PRUESS, J. F. BLOUNT & M. WEIGELE: Ro 22-5417, a new clavam antibiotic from *Streptomyces clavuligerus*. III. Absolute stereochemistry. J. Antibiotics 36: 217~225, 1983
5) BUSSON, R.; E. ROETS & H. VANDERHAEGHE: Chiroptical studies of penicillanates. *In* Recent Advances in the Chemistry of β-Lactam Antibiotics. *Ed.*, J. ELKS, pp. 304~313, Chem. Soc., London, 1977