Ro 22-5417, A NEW CLAVAM ANTIBIOTIC FROM *STREPTOMYCES CLAVULIGERUS*

II. FERMENTATION, ISOLATION AND STRUCTURE*

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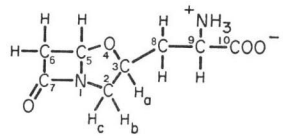
The purification of a new antibiotic, Ro 22-5417, was achieved by a variety of preparative column chromatographic methods. The antibiotic was obtained crystalline with an overall recovery of 3% after a 300-fold purification. The structure was determined from proton and ^{13}C NMR spectra to be 3-(7-oxo-1-aza-4-oxabicyclo[3.2.0]hept-3-yl)alanine.

In the preceding paper[1], the discovery and biological activities of the new antibiotic, Ro 22-5417, are reported. We describe here the large scale fermentation, details of the isolation procedure, and physical-chemical characterization of the substance which supports a proposed constitutional structure, 3-(7-oxo-1-aza-4-oxabicyclo[3.2.0]hept-3-yl)alanine (**I**). The stereochemistry of this new clavam antibiotic is presented in the succeeding report[2].

Structure of Ro 22-5417



I

Fermentation

Streptomyces clavuligerus NRRL 3585 maintained on starch - casein agar slants was used to inoculate 100 ml of TS$^+$ medium, containing in (g/liter): BBL Trypticase Soy Broth dehydrated (30), glycerol (10), in a 500-ml flask. The flask was incubated at 28°C on a rotary shaker for two days. Inoculum was prepared by transferring 10 ml of this growth into each of two 6-liter flasks, each of which contained 2 liters of TS$^+$ medium.

The 6-liter flasks were incubated at 28°C for 3 days on a rotary shaker (New Brunswick Scientific Co., Model G-52). Good results are obtained with incubation time from two to four days. The 4 liters of growth were then used to inoculate 230 liters of medium M-2, containing in (g/liter): glycerol (20), Soyalose 105 [Central Soya] (15), K_2HPO_4 (1), $CoCl_2 \cdot 6H_2O$ (0.01), SAG 4130 antifoam [Union Carbide] (0.1).

The fermentation in a 400-liter stainless steel fermentor was carried out at 28°C, with agitation at 150 rpm. The aeration rate was varied between 85 and 140 liters per minute to maintain the dissolved oxygen level above 50 percent of air saturation. The gauge pressure in the fermentor was 0.35 kg/cm^2. After four days of incubation, the broth (pH 7.2), which contained 20 to 50 mg antibiotic per liter, was harvested.

* Some of this work was presented at the 20th Interscience Conference on Antimicrobial Agents and Chemotherapy held in New Orleans, Louisiana, Sept. 22, 1980.

Isolation Procedure

The whole broth (190 liters) was cooled to 20°C, Darco G-60, 1.9 kg, (ICI-United States, Wilmington, Delaware) was added, and the mixture gently stirred for 15 minutes. After the pH was adjusted to 6.5 with 100 ml 5 N H_2SO_4, 6.8 kg of HyFlo Super Cel diatomaceous earth (Johns-Manville, Lompoc, California) was added, and solids were removed on a Bird rotary vacuum filter. The filter cake was washed with 22 liters tap water and the combined filtrate and wash was adjusted to pH 7.3 with 5 N H_2SO_4 and concentrated to a volume of 22 liters on a wiped film rotary vacuum evaporator (Model 08–032 TFP, Votator Div., Chemetron Corp., Louisville, Kentucky). The concentrate was then applied to a column (160 cm × 30 cm) of Amberlite XAD-2 resin (Rohm and Haas, Philadelphia, Pennsylvania), and the resin was eluted with deionized water (flow-rate 1.5 liters/minute). With the first appearance of salts in the effluent, seven 20-liter fractions were collected. The activity peak was found in the third fraction, but fractions 4~7 were more stable and hence more amenable to further purification. After lyophilization (651 M Vac-Pac, Hull Corp., Hatboro, Pennsylvania), 50~100 g solids were obtained from fractions 4~7 (purity 0.5~1.5% and recovery 10~25%).

Depending on the purity and stability of the crude preparation, a variety of procedures was used for further purification. The most reliable, albeit limited to a small scale, was as follows: Ten grams of solids were dissolved in about 30 ml of H_2O and filtered through a celite pad. The resulting clear amber solution (6.5 g solids in 38 ml) was chromatographed on the Waters Prep LC™/System 500 using a 370-gram Prep PAK-500/C_{18} column, with water elution. The activity, obtained in the second bed volume, 400 ml, was lyophilized to give 910 mg of solids.

A 900-mg portion of these solids was dissolved in water and chromatographed on Bondapak ™C_{18}/Porasil B (six *ca.* 61 by 0.95 cm columns in series) with water elution. After elution of one bed volume (approximately 90 ml), 5-ml fractions were collected. The peak bioactive fractions (28~31) were pooled and lyophilized to give 105 mg of a slightly yellow solid. A 100-mg portion of these solids was dissolved in 1 ml H_2O, diluted with 1 ml methanol and applied to the top of a Sephadex LH-20 column (50 cm × 2.5 cm). The column was eluted with 4 bed-volumes of methanol - water (1 : 1) and fifty 3-ml fractions were collected. The peak activity fractions (35~47) were pooled, concentrated to a small volume, and ethanol was slowly added. The solution was chilled to 4°C, after which a 17-mg crop of crystals was obtained by filtration; a second crop of 8 mg was recovered from the mother liquor (25% recovery: 3% overall).

In an alternative procedure, more suitable for larger scale work, a 17-g portion of solids obtained from fractions 4~7 of the previously described XAD-2 column was slurried in 80 ml water at pH 6.6. Insoluble material, mostly charcoal, was removed by filtration, and the combined filtrate and water wash (100 ml) was applied to the top of a column (50 cm × 4.2 cm) containing 700 ml AG50W X-4, 100~200 mesh, resin in the Na^+ form. The resin was then washed and eluted with distilled water at 5°C. The peak activity was obtained at an elution volume of 400~500 ml after the appearance of unretained substances in the effluent. Crystalline product was then obtained by evaporating this solution to a 2-ml volume followed by addition of 4 ml ethanol. However, when scaled up or when less pure starting material was used, it was necessary either to repeat the cation exchange resin procedure on a smaller scale or use chromatographic methods similar to those described in the previous paragraph.

Results and Discussion

As might be expected, Ro 22-5417 is a highly unstable molecule. The solution stability is shown in

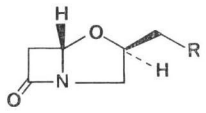
Fig. 1. Even greater instability is observed in more concentrated phosphate buffers. The dry crystalline substance, however, has shown no signs of decomposition after storage for 2 years at −20°C.

Elemental analysis was consistent with the molecular formula $C_8H_{12}N_2O_4$ (200.19).

Anal. Calcd.: C 47.99, H 6.04, N 13.99.
Found: C 47.30, H 6.04, N 13.74.

The optical rotation of the antibiotic is $[\alpha]_D^{25}$ −137.63° (*c* 0.76, H_2O), and mp 247~267°C (dec). The infrared spectrum (KBr disk) presented in Fig. 2 gave evidence for $-NH_3^+$ at 2960 cm^{-1}, β-lactam at 1760 cm^{-1} and COO^- at 1640 cm^{-1},

Proton NMR spectra in DMSO-d_6 and D_2O, which complemented each other very nicely, are presented and interpreted. The ^{13}C NMR spectrum was taken in D_2O (external TMS). The assignments based on these NMR spectra and extensive decoupling experiments are presented in



II R=OH
III R=CH_2OH

Fig. 1. Stability of Ro 22-5417 (10 μg/ml in 0.1 M phosphate-citrate buffers).

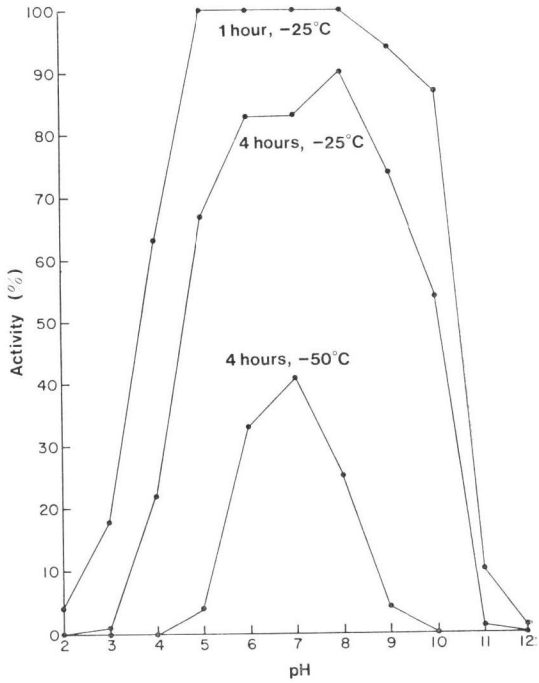


Table 1. These assignments are also consistent with those in the literature for corresponding positions in compounds II[3] and III[4], thus suggesting an analogous stereochemistry at the C-3

Fig. 2. IR Spectrum of Ro 22-5417.

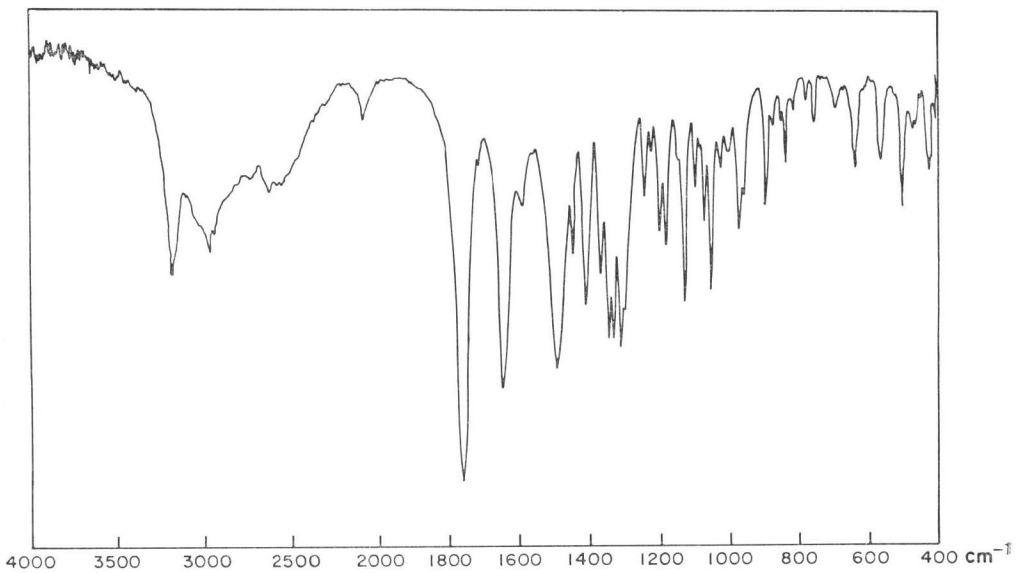


Table 1. NMR spectral assignments.

Carbon number*	^{1}H NMR				^{13}C NMR
	D_2O		DMSO-d_6		D_2O
	δ		δ		δ
8-CH_2	2.63	m, 2H, J=5 and 7Hz	1.75, 2.10	2ddd, 1H ea, J_{gem}=15Hz, J_{vic}=5 and 8Hz	34.1 (t)
2-CH_2	3.21	dd, 1H, J=11.5 and 7Hz	2.59	2dd, 1H ea, J_{gem}=11.5Hz	51.4 (t)
	4.49	dd, 1H, J=11.5 and 6Hz	3.85	J_{vic}=7.5 and 5.5Hz	
6-CH_2	3.40	d, 1H, J=16.5Hz	2.76	AB of ABX, 2H, J_{gem}AB=17Hz, J_{vic}AX<1, J_{vic}BX=3Hz	45.6 (t)
	3.83	dd, 1H, J=16.5 and 3Hz	3.33		
9-CH	4.39	t, 1H, J=5Hz	3.23	dd, 1H, J_{vic}=5 and 8Hz	53.9 (d)
3-CH	4.87	m, 1H	4.35	m, 1H	79.6 (d)
5-CH	5.94	d, 1H, J=3Hz	5.30	X of ABX, 1H, J_{vic}<1 and 3Hz	85.5 (d)
7 C=O					174.4 (s)[a]
10 C=O					182.6 (s)

[a] Assignment based on analogy to compound **III**[4].

* Numbering system is based on **I**.

and C-5 carbons. A more detailed discussion of the stereochemistry, including CD studies, is presented in the following paper[2].

References

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