[*R*-(*Z*)]-4-AMINO-3-CHLORO-2-PENTENEDIOIC ACID, A NEW ANTIBIOTIC

FERMENTATION, ISOLATION AND CHARACTERIZATION

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A new unsaturated glutamic acid analog, 4-amino-3-chloro-2-pentenedioic acid (ACPA) was isolated from a fermentation broth produced by a strain of *Streptomyces*. ACPA has a very narrow antibacterial spectrum, which is virtually limited to *Micrococcus luteus*.

In the course of screening for new broad spectrum antibiotics, it was found that a new strain of *Streptomyces viridogenes*[1] produced antibiotic activity with a wide spectrum of activity. Paper disc agar diffusion assays with *Micrococcus luteus* (ATCC 9341) and *Bacillus subtilis* (ATCC 6633) were used to follow the isolation of the antibiotic activity from fermentation filtrate. It soon became apparent that at least two antibiotics were present, *i.e.*, compound **I** with antibacterial activity only against the *Micrococcus* organism and compound **II** that had activity against both organisms. After purification, **I** was found to be 4-amino-3-chloro-2-pentenedioic acid (ACPA), a new unsaturated analog of chloro-glutamic acid and **II** was indicated by NMR to be identical to antibiotic FR-900148[2], a valine dipeptide of **I**. This report describes the fermentation, isolation and characterization of ACPA.

Fermentation

Fermentation studies with *Streptomyces viridogenes* (designated in the Merck Culture Collection as MA5450) attempted to maximize production of 4-amino-3-chloro-2-pentenedioic acid or FR-900148. Enriched production of ACPA was found when *S. viridogenes* was inoculated into a baffled 250-ml Erlenmeyer flask containing 50 ml of medium A (Table 1). Inoculates of medium A were incubated for 2 days at 28°C, 220 rpm on a gycorotary shaker (61-cm throw). Growth in medium A was then used to inoculate (5%) unbaffled 250-ml Erlenmeyer flasks containing 40 ml of medium B (Table 1). ACPA was initially detected after 1 day of incubation at 28°C, 220 rpm. This fermentation was complete after 5 days of incubation.

The compound FR-900148 was enriched when as above 2 days growth in medium A was used to inoculate (5%) 40 ml of medium C (Table 1) in an unbaffled 250-ml Erlenmeyer flask and incubating the flask for 2 days at 28°C, 220 rpm. The most consistent production of the antibiotic mixture occurred in 40 ml of medium D, (Table 1) inoculated (5%) with two days growth in medium A, incubated in an unbaffled 250-ml Erlenmeyer flask for 4 days at 28°C, 220 rpm.

Isolation Procedures

Compound I — ACPA

The purification procedure to isolate compound **I** is outlined in Fig. 1. The fermentation broth

Table 1. Composition of media.

Ingredient (g/liter)	Medium A	Medium B	Medium C	Medium D
Dextrose	1.0	45.0	20.0	—
Soluble starch	10.0	—	—	—
Corn meal	—	—	—	10.0
Beef extract	3.0	—	—	—
Yeast autolysate (Ardamine pH)	5.0	2.5	—	5.0
Peptone (NZ-Amine E)	5.0	—	—	—
Peptonized milk	—	24.0	—	—
Cottonseed meal (Pharmamedia)	—	—	6.0	—
Tomato paste	—	—	—	30.0
$(NH_4)_2SO_4$	—	—	4.0	—
KH_2PO_4	0.182	—	—	—
Na_2HPO_4	0.190	—	—	—
$MgSO_4 \cdot 7H_2O$	0.05	—	—	—
$CaCO_3$	0.5	—	8.0	—
Polyglycol P2000	—	2.5 ml	—	—
pH	7~7.2	7.0	7.0	5.0

(13 liters) was adjusted to pH 3.0 with 2.5 N HCl and filtered. The filtrate (121 g solids) contained both *B. subtilis* (ATCC 6633) and *M. luteus* (ATCC 9341) activity and was treated with 250 g activated carbon. The slurry was filtered. Most of the *Micrococcus* activity was in the carbon filtrate whereas the *Bacillus* activity was reduced by one-half. The filtrate was passed through an 800 ml Dowex 50-X12 (H^+ cycle) resin column followed by one liter of water. The resin spent effluent was devoid of bioactivity. The resin was eluted with 10 liters of 2% pyridine and one liter fractions were taken. The initial eluate fractions were at pH 3.0 and contained only the *Micrococcus* activity. The four highest activity fractions were combined and evaporated to 50 ml (1.7 g solids). An equal volume of methanol was added and the mixture was put in the refrigerator for 16 hours. The crystals formed were filtered off and dried to yield 643 mg of product. The crystals were subjected to ^1H NMR studies.

Fig. 1. Isolation procedure for compound **I**.

Fermentation broth
↓ Acidified with HCl to pH 3
Filtrate
↓ Treated 2% carbon
Carbon filtrate
↓ Dowex 50-X12 (H^+) eluted 2% pyridine
Rich eluate
↓ Free acid evaporated to 40 mg/ml
Concentrate
↓ Crystallized with methanol
Crystals (0.64 g)

Forty mg of crystals were dissolved in one ml H_2O, heated and allowed to cool to room temperature to initiate large crystal formation. The solution was then put in the refrigerator for 72 hours. The large crystals formed were evaluated by X-ray crystallography.

Compound **II** — FR-900148

The isolation was monitored by both *B. subtilis* and *M. luteus* agar diffusion assays as above. Fermentation broth was centrifuged. The clear liquid (900 ml; 19.8 g solids) was adjusted to pH 9.0 and passed through a 175-ml Dowex 1-X2 (Cl^-) resin column followed by 400 ml H_2O. The adsorbed antibiotic was eluted with 800 ml of 3% NaCl, taking 200-ml fractions. The active fractions were combined, adjusted to pH 7.0 and evaporated to 45 ml. The concentrate was treated with an equal volume of methanol to precipitate salt and the clear liquid was evaporated to 30 ml. Twenty

ml of the concentrate was passed through a 900-ml Biogel P-2 column collecting 10-ml fractions. The first peak of *Bacillus* activity occurred in fraction No. 54. It was equally active against *M. luteus* and *B. subtilis*. Later fractions contained increased ratios of *Micrococcus* activity. Fraction No. 54 was freeze-dried to yield 38 mg of product which was subjected to NMR analysis.

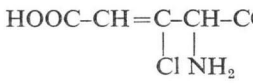
Physico-chemical Properties

Compound I — ACPA

The crystals decomposed above 138°C. The antibiotic is not extracted from water with organic solvents.

Anal Calcd for $C_5H_6NO_4Cl$: C 33.42, H 3.37, N 7.80, Cl 19.75
Found: C 33.55, H 3.38, N 7.70, Cl 19.24

The ^1H NMR spectrum on a Varian SC300 showed only two peaks in D_2O (two drops of DCl added) with the following characteristics: δ 5.17 (1H, s) and 6.80 (1H, s) consistant with a structure of:



X-Ray Crystallography

Crystals of ACPA formed from water in space group $P2_1$ with a=5.178(2)Å, b=7.742(4), c=9.175(3) and β=100.43(3)° with Z=2. Out of 532 unique reflections measured with a Nicolet $P2_1$ X-ray diffractometer using CuKα radiation 514 were observed ($I \geq 3\sigma I$). The structure was solved with application of direct methods[3] and refined by minimizing $\Sigma\omega(|F_o|-|F_c|)^2$ with $\omega=1/(\sigma F_o)^2$. At the end of anisotropic temperature parameter refinement of the non-hydrogen atoms, anomalous scattering contributions were introduced to determine the absolute configuration. The R enantiomer refined to a residual value (R factor) of 0.0690 while the S enantiomer refined to 0.0717 which is statistically significant at the 99.5% level[4]. This assignment was confirmed by careful remeasurement of 10 enantiomorph sensitive reflections and their Friedel pairs. Figs. 2[5] and 3 show the correct configuration of C4 of R which is analogous to the S configuration of L-amino acids. In addition, the X-ray experiments show that the configuration around the double bond is Z. The final R factor when isotropic hydrogens were added and refined was 0.057. Tables containing the X-ray coordinates and temperature parameters will be deposited with the Cambridge Crystallographic Data Center. As would be expected ACPA exists in a zwitterionic form with the δ carboxyl protonated.

Fig. 2. A computer generated perspective drawing of [*R*-(*Z*)]-4-amino-3-chloro-2-pentenedioic acid (ACPA) showing the correct absolute configuration.

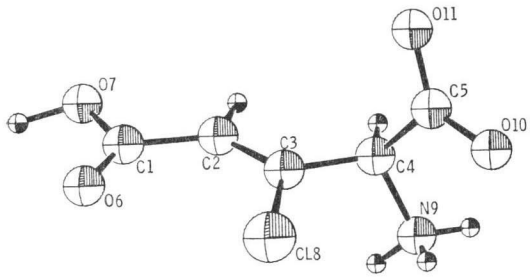
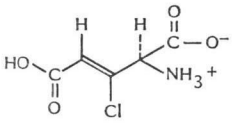


Fig. 3. ACPA.



Compound II — FR-900148

A D_2O ^1H NMR spectrum using a Varian XL-200 showed the following characteristics for **II**: δ 1.06 (3H, d, J=7 Hz), 1.07 (3H, d, J=7 Hz), ~2.3 (1H, m), 3.96 (1H, d, J=6 Hz), 5.02 (1H, s), 6.48 (1H, s). These peaks are consistent to those reported for FR-900148.

Discussion

The discovery of 4-amino-3-chloro-2-pentenedioic acid was fortuitous in that only the *Micrococcus luteus* organism out of fifty Gram-positive and Gram-negative organisms tested revealed its antibiotic activity. Its presence in the fermentation possibly acts as a precursor for the formation of FR-900148, the valine dipeptide of the above compound, which has wide-spectrum and cell wall antibiotic activity.

References

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