FR-900130, A NOVEL AMINO ACID ANTIBIOTIC

II. ISOLATION AND STRUCTURE ELUCIDATION OF THE ACETYL DERIVATIVE OF FR-900130

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FR-900130 is a new antibiotic having an acetylene moiety and is very unstable in aqueous solution. The antibiotic was isolated as the acetyl derivative by ion-exchange resin and adsorption chromatography. The structure has been determined to be L-2-amino-3-butynoic acid by spectral data of its derivatives.

A new antibiotic FR-900130 shows synergy with D-cycloserine[1]. Although it is unstable especially in concentrated solution, the antibiotic powder (70% pure) was obtained in a low yield as described in the preceding paper[1]. The properties of the antibiotic as its acetyl derivative were examined. The acetyl derivative was found to be stable enough to be isolated by usual methods. This paper describes isolation procedures of the acetyl derivative of FR-900130 in a good yield by acetylation of the crude sample obtained from the filtrate. Agar plate supplemented with the supernatant from kidney homogenate of rat was successfully used to measure its activity. The spectral data of the acetyl, acetylmethyl, hydrogenated derivatives and unexpected by-products are discussed.

Isolation of the Acetyl Derivative of FR-900130

The diagram of purification method described below is shown in Fig. 1. The filtrate (240 liters) was adsorbed onto Duolite C-20 (H^+ cycle, 36 liters). The column was washed with water and eluted with 150 liters of 0.5 N sulfuric acid. The eluate was neutralized to pH 6.0 with barium hydroxide and the precipitate formed was discarded. The supernatant was concentrated to a volume of 5 liters and was treated with 150 g of activated carbon to remove impurity. Then the decolorized concentrate was poured into the same portion of methanol and the precipitate formed was filtered off. The above-mentioned operation was monitored using the usual *Staphylococcus* agar plate. After this stage of purification, deacetylase-agar plate was used to determine the antibiotic content.

To 10 liters of 50% aqueous methanol solution of FR-900130 was added acetic anhydride (180 ml) until a ninhydrin test showed a negative reaction. The solvent was evaporated under reduced pressure to a volume of 3 liters and the concentrate containing the acetyl derivative of FR-900130 was adjusted to pH 1.4 with 6 N hydrochloric acid solution. The acidified solution was treated with the same volume of ethyl ether in order to remove free acetic acid, and then the acetylated antibiotic was extracted three times with the same portion of *n*-butanol. The butanol extracts were combined and the acetylated antibiotic was retransferred to an aqueous phase using water of pH 7.0. The aqueous layer separated was adjusted to pH 1.4 and was passed through a column of activated charcoal (2 liters). The column, after washing with water, was eluted with 70% aqueous methanol. The eluate was adjusted to pH 7.0 and was evaporated *in vacuo*. The residue was chromatographed on a column

of silicagel G (500 ml) and the elution of the antibiotic was carried out with a solvent of chloroform - methanol (1:1). The active fractions were concentrated and adjusted to pH 1.4. Then, it was applied to a column of silicic acid with an eluant of a mixture of chloroform - ethyl acetate (1:2). The fractions containing the acetylated antibiotic were combined and neutralized to pH 6.5 with 1 N sodium hydroxide solution. The evaporation of the eluate gave 1.7 g of the pure acetyl derivatives of FR-900130.

Fig. 1. Isolation procedure of the acetyl derivative of FR-900130.

Filtrate
↓ acidified to pH 2
Duolite C-20 (H^+ cycle)
↓ eluted with 0.5 N H_2SO_4, neutralized with $Ba(OH)_2$
treated with activated carbon, added methanol
Acetylation with acetic anhydride
↓ concentrated to aqueous solution, adjusted to pH 1.4
removal of free acetic acid with ethyl ether
Extraction of the acetyl derivative with *n*-butanol
↓ transferred to aqueous phase at pH 7
acidified to pH 1.4
Carbon column
↓ eluted with 70% aqueous methanol
Silica gel column
↓ eluted with chloroform - methanol (1:1)
concentrated and adjusted to pH 1.4
Silicic acid column
↓ eluted with chloroform - ethyl acetate (1:2)
Active fraction
↓ adjusted to pH 6.5
Freeze-dry
↓
White powder (Acetyl derivative of FR-900130)

Characterization and Structure

The acetyl derivatives of FR-900130 (**II**) is a white powder, easily soluble in water, methanol, and acetone. Rf values of thin-layer chromatography are 0.5 on cellulose (70% aqueous propanol) and 0.08 on silica gel (chloroform - methanol, 2:1). It shows a negative ninhydrin reaction although the original antibiotic shows a unique ninhydrin color reaction, which suggests an unusual amino acid. IR spectrum is shown in Fig. 2. A sharp band at 3300 cm^{-1} and a band at 2150 cm^{-1} indicate the probable presence of a terminal acetylene group. The

Fig. 2. IR spectrum of the acetyl derivative of FR-900130 (KBr).

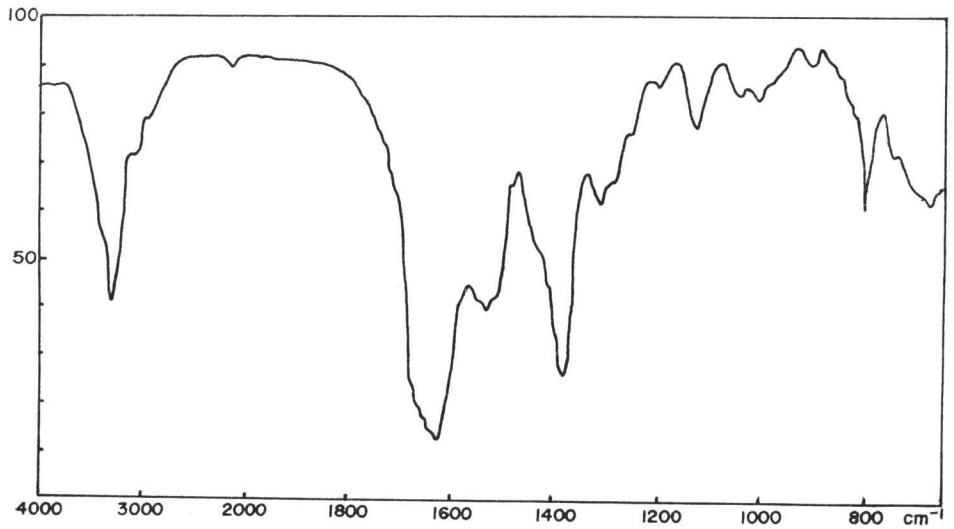
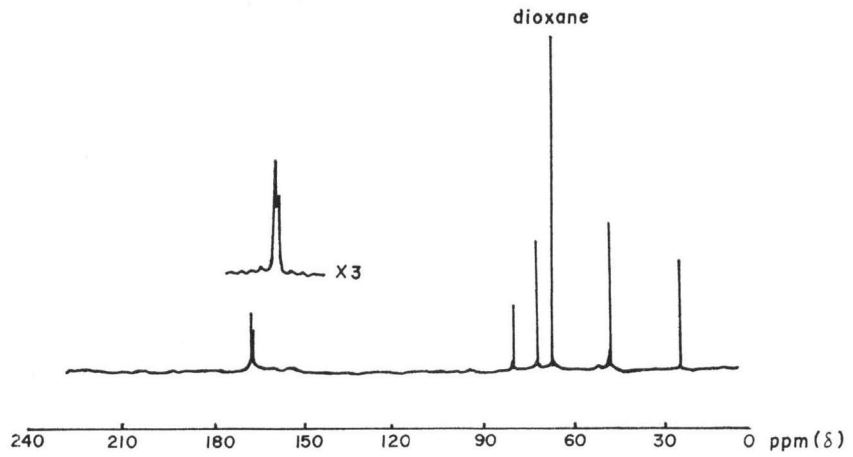


Fig. 3. ^{13}C-nmr spectrum of the acetyl derivative of FR-900130 (D_2O).



molecular formula of **II** was established to be $C_6H_6NO_3Na \cdot \frac{1}{2}H_2O$ from elemental analysis and titration. (Calcd. for $C_6H_6NO_3Na \cdot \frac{1}{2}H_2O$: C 41.86, H 4.07, N 8.14, Na 13.37, found: C 41.67, H 4.21, N 8.45, Na 12.30, equivalent weight 180). The ^{13}C-nmr data of **II**, shown in Fig. 3 and Table 1, also supported the proposed formula. The ^{1}H-nmr spectrum shows N-acetyl protons at δ 1.85 (3H, s), one proton at δ 2.80 (1H, d, J=3 Hz, CH≡C–), one proton on α carbon of an amino acid at δ 4.58 (1H, dd, J=3 Hz, 8 Hz, CO–NH–CH–COONa) and exchangeable proton of amide at δ 7.78 (1H, d, J=8 Hz) (Fig. 4). The above-mentioned protons could be assigned as follows: HC≡C–CH–HN–CO–CH_3, with long distance coupling. The optical rotation of **II** is $[\alpha]_D^{20}$ +37.5° (*c* 0.5, H_2O).

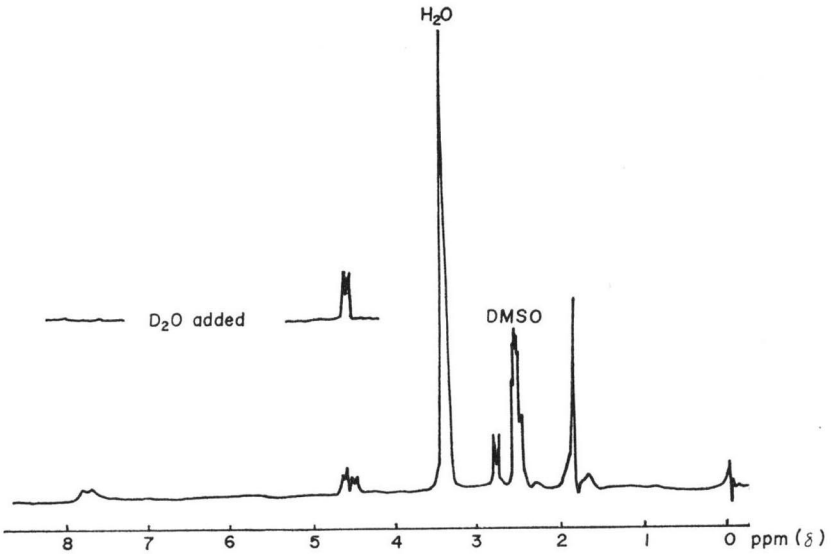
Table 1. ^{13}C-nmr data of the acetyl derivative of FR-900130.

Chemical shift[1]	Off-resonance[2]	Assignment[3]
22.7	q	CH_3CO–
47.4	d	–N–CH–CO–
73.3	d	H–C≡C–
80.7	s	H–C≡C–
173.7	s	–COONa
173.9	s	–CO–CH_3

1) δ value in ppm relative to TMS using dioxane as internal standard
2) Multiplicity: q=quartet, d=doublet, s=singlet
3) Refer to the structure **II** in Scheme 1.

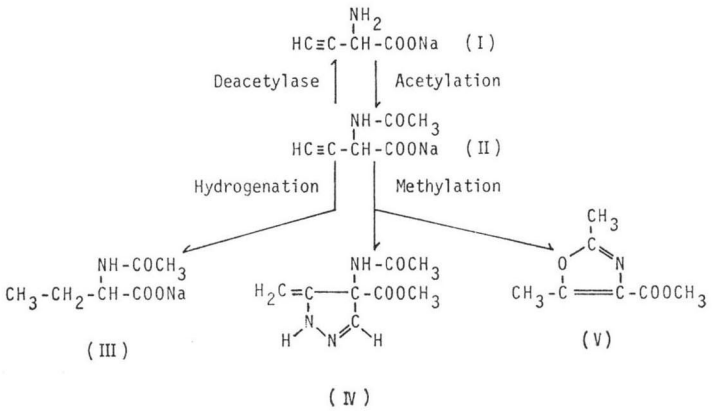
Catalytic hydrogenation gave the product **III**, which was identified as N-acetyl-α-aminobutyric acid from the ^{1}H-nmr spectrum and from the Rf value of an authentic sample prepared chemically. By comparing the optical rotation of **III** with that of a chemically synthesized sample, **III** has been determined to be L-configuration. In order to confirm the molecular weight of **II** by mass spectroscopy, **II** was methylated with diazomethane. As a result, two products **IV** and **V** were obtained as crystals. **IV** is yellow-colored and the molecular formula $C_5H_{11}N_3O_3$ was established by elemental analysis and mass spectrometry (*m/e* 197 M^+). The nmr spectral data along with other data described in Experimental suggested that **II** formed the pyrazole derivative (**IV**) by treatment with diazomethane (Scheme 1). The ^{1}H-nmr spectrum of **V** unexpectedly showed only three singlets due to methyl protons at δ 2.40, δ 2.55 and δ 3.86. The molecular formula was determined as $C_7H_9NO_3$. This suggested that **II** formed the oxazole (**V**) by intramolecular ring closure between the acetylene and the acetyl carbonyl function. This information indicates that **II** has a propargyl moiety in the molecule.

Fig. 4. ^{1}H-nmr spectrum of the acetyl derivative of FR-900130 (DMSO/D_2O).



Finally, compound **II** was incubated with the deacetylase from rat kidney. It has been confirmed that **II** was deacetylated and converted to the naturally occurring FR-900130 (**I**) by bioautography of the incubation mixture. The aforementioned results are summarized in Scheme 1. Consequently, FR-900130 was determined to be L-2-amino-3-butynoic acid.

Scheme 1.



Experimental

(1) Hydrogenation

A 200 mg sample of **II** was dissolved in 20 ml of water and added with 50 mg of palladium-carbon. The mixture was shaken under one atmosphere pressure of hydrogen. Two moles of hydrogen was absorbed in 30 minutes. The catalyst was filtered off and washed with water. The filtrate and the wash were concentrated to dryness to yield 180 mg of white powder (**III**).

IR (nujol), 3300, 2900, 1700~1500, 1410, 1300, 1150, 810 cm^{-1}
nmr (DMSO), δ 0.76 (3H, t, J=6 Hz), δ 1.60 (2H, m), δ 1.84, (3H, s), δ 3.93 (1H, m), δ 7.50 (1H, d, J=7 Hz)
$[\alpha]_D^{20}$ −7.2 (*c* 1, H_2O)
Calcd. for $C_6H_{10}NO_3Na\cdot\frac{1}{2}H_2O$, C 40.91, H 6.25, N 7.95, Na 13.05
Found, C 41.59, H 6.13, N 8.49, Na 12.15

(2) Methylation

A 200 mg sample of **II** was dissolved in 20 ml of water (pH 1.4) and was adsorbed on a 50 ml of

charcoal. The column was eluted with 50% aqueous methanol. The eluate containing the free acid of **II** was methylated with diazomethane in ether at 20°C. Two kinds of products were detected on the thin-layer chromatography in the reaction mixture. It was concentrated *in vacuo* and the residue was purified on a silica gel column. Fractions eluted with the solvent chloroform - ethyl acetate (4: 1) gave 90 mg of colorless crystals (**V**). Fractions eluted with ethyl acetate gave 80 mg of yellow crystal (**IV**).

IV IR (nujol): 3400, 3250, 3010, 2930, 2830, 1750, 1630, 1530, 1450, 1430, 1380, 1300, 1250, 1075, 1005, 970, 860, 820, 770, 720, 690 cm^{-1}

nmr (d_6-acetone): δ 1.93 (3H, s), δ 3.70 (3H, s), δ 5.50 (1H, s), δ 7.10 (1H, s), δ 7.40 (H, broad s, exchangeable), δ 7.95 (1H, broad s, exchangeable)

UV (H_2O): 282 nm (ε 4160)

Mass: *m/e* 197 (M^+)

Calcd. for $C_8H_{11}N_3O_3$: C 48.72, H 5.62, N 21.31

Found: C 48.30, H 5.70, N 22.82

V IR (nujol): 2930, 2850, 1720, 1625, 1600, 1440, 1420, 1350, 1220, 1200, 1100, 975, 930, 820, 780, 745, 670 cm^{-1}

nmr ($CDCl_3$): δ 2.40 (3H, s), δ 2.55 (3H, s), δ 3.86 (3H, s)

Mass: *m/e* 155 (M^+)

Calcd. for $C_7H_9NO_3$: C 54.19, H 5.85, N 9.05

Found: C 54.16, H 5.84, N 9.55

(3) Deacetylation by enzyme

An 1 mg/ml sample of **II** in water was mixed with the supernatant of rat kidney homogenate at 37°C for 2 hours. An aliquot of the reaction mixture was spotted on a tlc plate (cellulose) and the culture filtrate of No. 4707 (containing FR-900130) was spotted adjacent to the spot. The antibiotic was detected by bioautography with a solvent system of 70% aqueous propanol on a cellulose plate (Eastman).

Discussion

This is the first report on the microbial production of L-2-amino-3-butynoic acid, although a chemical synthesis of this compound has been described as a corrosion inhibitor[2]. FR-900130 has a molecular formula identical with azirinomycin[3,4] but is distinct in its chemical structure and other properties; chromatographic behavior, extraction into ethyl acetate. FR-900130 is an antibiotic with cell wall-inhibitory activity, but its instability diminishes its practical utility. Chemical manipulation of its structure would be needed for the further development of this antibiotic.

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