A NEW ANTITUMOR ANTIBIOTIC, FR900840

II. STRUCTURAL ELUCIDATION OF FR900840

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The structure of FR900840, a new antitumor antibiotic produced by a strain of *Streptomyces* has been deduced as **1** on the basis of spectroscopic and chemical evidence and finally confirmed by synthesis from L-threonine and L-serine.

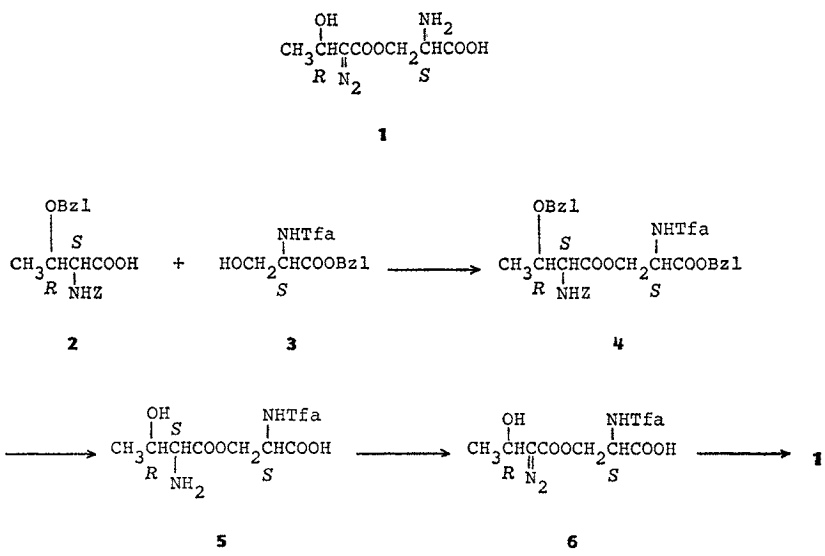
FR900840 (**1**) is a new antibiotic with potent antitumor activity. Its discovery, isolation, and characterization were described in the preceding paper of this series.[1] In this paper, we report the structural elucidation and the synthesis of this antibiotic.

FR900840 (**1**), $C_7H_{11}N_3O_5$, mp 123~125°C (dec), $[\alpha]_D^{23}$ +1.5° (*c* 1.0, H_2O, pH 7.0), was isolated as a major compound from the fermentation broth of *Streptomyces* sp. No. 8727. Its molecular formula was established on the basis of the mass spectral data and the elemental analysis (see Experimental). The antibiotic showed an IR absorption band at 2160 cm^{-1} (Nujol) and a UV absorption maximum at 257.5 nm (H_2O, pH 7.0), consistent with the presence of a diazo function conjugated with an ester group in the molecule (see ref 2). This presumption was also supported by the fact that dissolution of the antibiotic in 0.1 N HCl brought about gas evolution (provably N_2), resulting in the disappearance of the characteristic IR and UV absorbances.

FR900840 was positive to the ninhydrin test and showed signals at 4.62 (2H, d, J=3.8 Hz) and 4.10 (1H, t, J=3.8 Hz) in the ^1H NMR spectrum (D_2O, pD 7.0), suggesting the presence of a serine structure in the molecule. This was confirmed by treating the antibiotic with 6 N HCl (reflux) to generate L-serine, which was identified with an authentic sample on HPLC coupled with a chiral derivation method using 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl isothiocyanate (GITC).[3]

The ^1H NMR spectrum of FR900840 also showed signals attributable to the partial structure of $CH(OH)CH_3$ at 4.87 (1H, q, J=6.6 Hz) and 1.42 (3H, d, J=6.6 Hz). Combining the known structure features suggested this group was linked to the diazo ester group described above and was biogenetically derived from threonine. For confirmation, the antibiotic was subjected to hydrogenation over Pt in phosphate buffer at pH 7.2, followed by hydrolysis in refluxing 6 N HCl. Analysis of the hydrolysate on HPLC coupled with the GITC method[3] revealed that L-threonine and/or D-*allo*-threonine were obtained together with L-serine. L-Threonine and D-*allo*-threonine had the same retention time (24.08~24.28 minutes) under the HPLC conditions we adopted. D-Threonine and L-*allo*-threonine were not detected in the hydrolysate (D-threonine 28.12 minutes; L-*allo*-threonine 22.86 minutes). Since L-threonine and D-*allo*-threonine have the same configuration (*R*) at the hydroxy-bearing carbons, the corresponding carbon in the antibiotic was in any event concluded to be the *R*-configuration.

The data described above for FR900840 were in good agreement with the structure **1**. This was confirmed by synthesis from L-threonine and L-serine as follows.



Bzl: $CH_2C_6H_5$, Z: $COOCH_2C_6H_5$, Tfa: $COCF_3$.

O-Benzyl-*N*-carbobenzyloxy-L-threonine (**2**) (Kokusan Chemical Works) was converted to the mixed anhydride *in situ* using pivaloyl chloride and coupled to *N*-trifluoroacetyl-L-serine benzyl ester (**3**)[4] to yield the ester **4** (66%). Deprotection of the benzyl and carbobenzyloxy groups in **4** by hydrogenolysis using 10% Pd-C gave the amino acid **5** (94%). This compound was then treated with KNO_2 in H_2O containing chloroacetic acid and the product **6** was subsequently treated with Acylase I (Sigma Chemical Company) at room temperature affording, after purification by chromatography on charcoal and silica gel, the subject compound **1** (26%), which was identical with the natural product in all respects.

The structure of FR900840 was thus established as being **1**. FR900840 belongs to a family of antibiotics possessing the diazo functional group such as azaserine[5] and 6-diazo-5-oxo-L-norleucine.[6] The detailed biological activity of FR900840 is described in the following papers of this series.[7]

Experimental

IR spectra were recorded on a Jasco A-102 spectrophotometer. UV spectra were measured on a Hitachi 220A spectrophotometer. ^1H and ^{13}C NMR spectra were recorded by using Bruker AM200 and AM400wb spectrometers. Fast atom bombardment (FAB)-MS were measured on a VG ZAB-SE spectrometer. TLC was carried out on Silica gel 60 F_{254} plates (E. Merck AG) using solvent systems of BuOH - AcOH - H_2O (4:1:2) (solvent A) and BuOH - EtOH - $CHCl_3$ - NH_4OH (4:7:2:7) (solvent B): The spots were detected by ninhydrin. HPLC was performed on a column (4.6×250 mm) of TSK gel ODS-80Tm (Tosoh Manufacturing Co., Ltd.): Elution was by 0.1% aq H_3PO_4 - MeOH (9:1)/MeCN - MeOH (9:1) using a linear gradient from 8:2 to 6:4 within 60 minutes; flow rate, 1.0 ml/minute; detection, UV 250 nm.

FR900840 (1)

For isolation of FR900840, see Materials and Methods section in ref 1: MP 123~125°C (dec); $[\alpha]_D^{23}$ +1.5° (*c* 1.0, H_2O, pH 7.0); IR ν_{max} (Nujol) cm^{-1} 3430, 3270, 2160, 1660 (br), 1610 (br); UV $\lambda_{max}^{H_2O}$ (pH 7.0) nm (ε) 257.5 (13,000); ^1H NMR (D_2O, pD 7.0) δ 4.87 (1H, q, J=6.6 Hz), 4.62 (2H, d, J=3.8 Hz), 4.10 (1H, t, J=3.8 Hz), 1.42 (3H, d, J=6.6 Hz); ^{13}C NMR (D_2O, pD 7.0) 173.55 (s), 169.99 (s), 66.15 (t), 64.74 (d), 56.67 (d), 22.15 (q); FAB-MS *m/z* 218 (M^++1).

Anal Calcd for $C_7H_{11}N_3O_5 \cdot H_2O$: C 35.74, H 5.57, N 17.87.
Found: C 35.99, H 5.58, N 17.65.

Acid Hydrolysis of FR900840 (**1**)

FR900840 (10 mg) was dissolved in 6 N HCl (5 ml) and heated for 20 hours at 110°C. The reaction mixture was evaporated *in vacuo* to give a crude mixture: TLC, Rf 0.33 (solvent A) and 0.36 (solvent B). These Rf values were identical with those of serine. The crude mixture was treated with GITC according to the method reported in the literature[3] and identified as L-serine by HPLC: Retention time 20.83 minutes (L-serine 20.86 minutes; D-serine 21.84 minutes).

Hydrogenation of FR900840 (**1**)

A solution of **1** (100 mg) in phosphate buffer (pH 7.2) was hydrogenated over Pt in the usual manner. After removal of the catalyst by filtration, the filtrate was purified by chromatography on charcoal (200 ml) eluting with H_2O to give the pure hydrogenated product (90 mg): TLC, Rf 0.2 (solvent A); IR (KBr) cm^{-1} 3300, 1730; ^1H NMR (D_2O) 4.75~4.50 (3H, m), 4.30~4.05 (2H, m), 1.40~1.10 (3H, m).

Acid Hydrolysis of the Hydrogenated Product of FR900840 (**1**)

The hydrogenated product (10 mg) of **1** was dissolved in 6 N HCl (5 ml) and heated for 20 hours at 110°C. The reaction mixture was evaporated *in vacuo* to give a crude mixture: TLC, Rf 0.33 and 0.37 (solvent A), and 0.36 and 0.45 (solvent B). These Rf values were identical with those of serine and threonine, respectively. The crude mixture was treated with GITC according to the method reported in the literature:[3] HPLC showed two peaks, retention time 21.03 and 24.27 minutes. The former peak was identified with that of L-serine (20.86 minutes; D-serine 21.84 minutes). The latter peak was identical with those of L-threonine and/or D-*allo*-threonine (L-threonine 24.08 minutes; D-*allo*-threonine 24.28 minutes; D-threonine 28.12 minutes, L-*allo*-threonine 22.86 minutes).

Coupling of the L-Threonine and L-Serine Derivatives **2** and **3** to Compound **4**

A solution of pivaloyl chloride (1.22 ml, 10 mmol) in anhydrous EtOAc (5 ml) was added dropwise to a solution of *O*-benzyl-*N*-carbobenzyloxy-L-threonine (**2**) (3.40 g, 10 mmol) and *N*-methylmorpholine (1.90 ml, 10 mmol) in anhydrous EtOAc (10 ml) with stirring at −15°C. After stirring for an additional 30 minutes at the same temperature, the mixture was cooled to −78°C and a solution of *N*-trifluoroacetyl-L-serine benzyl ester (**3**)[4] was added dropwise with stirring. The temperature of the reaction mixture was gradually raised to room temperature and the mixture was stirred overnight. The mixture was washed successively with 2 N HCl, H_2O, aq $NaHCO_3$ (saturated), H_2O, and brine, and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* left a residue (6 g), which was purified by column chromatography on silica gel eluting with $CHCl_3$ to give pure compound **4** (4.0 g, 66%) as an oil: IR ($CHCl_3$) cm^{-1} 3400, 3000, 1740, 1720; ^1H NMR ($CDCl_3$) δ 7.65 (1H, d, J=8 Hz), 7.40~7.20 (15H, m), 5.43 (1H, d, J=8 Hz), 5.20 (2H, s), 5.17 (1H, d, J=12 Hz), 5.08 (1H, d, J=12 Hz), 4.88 (1H, dt, J=8 and 3 Hz), 4.68 (1H, dd, J=11.5 and 3 Hz), 4.50 (1H, d, J=11.5 Hz), 4.47 (1H, dd, J=11.5 and 3 Hz), 4.46 (1H, d, J=11.5 Hz), 4.24 (1H, dd, J=8 and 3 Hz), 4.03 (1H, dq, J=3 and 7 Hz), 1.20 (3H, d, J=7 Hz); FAB-MS m/z 617 (M^++1); $[\alpha]_D^{23}$ −15.0° (c 1.0, MeOH).

Removal of the Benzyl and Carbobenzyloxy Groups of **4** to Compound **5**

Compound **4** (500 mg) was dissolved in AcOH (10 ml) and hydrogenated over 10% Pd-C (250 mg) in the usual manner (3 atm of H_2). After removal of the catalyst by filtration, the filtrate was evaporated *in vacuo* and the residue was dissolved in MeOH (small amount) and triturated with Et_2O to give a precipitate which was crystallized from MeOH - H_2O to yield pure compound **5** (230 mg, 94%): IR ν_{max} (Nujol) cm^{-1} 3600~2400, 1740, 1700; ^1H NMR (D_2O) δ 4.72 (1H, dd, J=13 and 5 Hz), 4.52~4.48 (2H, m), 4.23 (1H, dq, J=4 and 7 Hz), 3.77 (1H, d, J=4 Hz), 1.26 (3H, d, J=7 Hz), 3.77 (1H, d, J=4 Hz), 1.26 (3H, d, J=7 Hz); FAB-MS m/z 303 (M^++1); $[\alpha]_D^{23}$ +7.0 (c 1.0, H_2O).

Conversion of **5** to FR900840 (**1**) *via* **6**

4 M Chloroacetic acid in H_2O (20 ml) and 6 M KNO_2 in H_2O (120 ml) were added to a solution of compound **5** (109 mg) in H_2O (10 ml) and the mixture was stirred at room temperature. After 15

minutes, Acylase I (20 mg, Sigma A 7264) was added to the above mixture (**6**) and the pH was adjusted to 7.0 by adding 2 M Tris buffer (pH 8.0). The mixture was then stirred at room temperature for 120 minutes during which time the pH was maintained at 7.0~7.3. The mixture was lyophilized and the residue was purified by column chromatography on charcoal (100 ml) eluting with Me_2CO - H_2O (5:95). The eluate was concentrated *in vacuo* and the residue was further purified by column chromatography on silica gel (10 ml) eluting with $CHCl_3$ - MeOH - H_2O (5:3:1). Evaporation of the solvent *in vacuo* gave a residue which was crystallized from EtOH to yield pure compound **1** (20 mg, 26%): The product **1** was identical with the natural product by IR, UV, ^1H NMR, and MS spectra and also in biological activities.

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