PRECURSORS IN THE BIOSYNTHESIS OF FR-900482, A NOVEL ANTITUMOR ANTIBIOTIC PRODUCED BY *STREPTOMYCES SANDAENSIS*

TAKASHI FUJITA, SHIGEHIRO TAKASE, TAKANAO OTSUKA, HIROSHI TERANO and MASANOBU KOHSAKA

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan

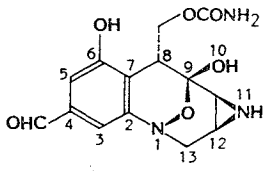
(Received for publication November 2, 1987)

A new antitumor antibiotic FR-900482 (**1**), isolated from the culture broth of *Streptomyces sandaensis* No. 6897, was found to have unique structure[1,2]. FR-900482 differs from the mitomycin family of antibiotics due to the lack of a quinoid structure, although FR-900482 resembles the mitomycins with respect to the aziridine function and a carbamoyloxymethyl group. Examination of the structure suggests that the biosynthesis of FR-900482 may be presumed to be derived from a number of compounds which structurally resemble parts of the molecule.

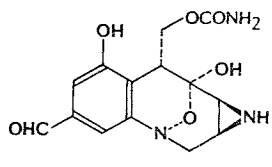
Recently, it has been reported that 3-amino-5-hydroxybenzoic acid (AHBA) is involved in the biosynthesis of antibiotics, actamycin[3], rifamycin B[4], geldanamycin[5] and mitomycins[6]. The ansamycins and the mitomycins contain a seven carbon amino unit, derived from AHBA. Furthermore, HORNEMANN and AIKMAN showed that in feeding D-[1-^{14}C,^{15}N]glucosamine to *Streptomyces verticillatus*, ^{14}C was incorporated into mitomycin B and the ^{15}N was incorporated into the aziridine ring to a similar extent and in direct relation to the amount of precursor added. They suggest that both isotopes are predominantly incorporated without separation[7]. We present here the experiments probing the role of AHBA and D-glucosamine on FR-900482 biosynthesis.

S. sandaensis No. 6897 was inoculated into a seed medium (40 ml) in a 250-ml Erlenmeyer flask and cultured at 30°C for 72 hours at 250 rpm using a rotary shaker. The seed medium (pH 7.0) was composed of soluble starch 2%, glucose 0.5%, dried yeast 1%, Pharmamedia 1%, corn steep liquor 0.5% and $CaCO_3$ 0.2%. The seed culture (1 ml) was transferred to 50 ml of a production medium and cultured for 144 hours under the same conditions. The production medium (pH 6.0) was composed of soluble starch 2%, maltose 2%, dried yeast 1%, wheat germ 3% and $CoSO_4 \cdot 7H_2O$ 1×10^{-3}%. Radioactive compounds were added to fermentation broths at 72 hours to give the final concentration indicated in Table 1. After 72 hours of additional incubation, the fermentation was terminated and the mycelia were removed by centrifugation (2,000 rpm for 15 minutes). The supernatant fluid was passed through a column of Diaion HP-20 and washed with deionized water. The column was eluted with 50% aq MeOH. The eluate was subjected to TLC on silica gel plates in $CHCl_3$ - MeOH (4:1) (Rf value: 0.20 and 0.45) or BuOH - AcOH - H_2O (20:1:2) (Rf value: 0.50) and the resulting chromatogram was scanned for radioactivity using a radioactive scanner. The minimum detectable incorporation of radioactive precursors into FR-900482 is approximately 0.1%.

As shown in Table 1, D-[*U*-^{14}C] or D-[1-^{14}C]-glucosamine and [7-^{14}C]AHBA were effectively incorporated into FR-900482. Further, fermentation medium supplemented with D-glucosamine (0.1~5.0 mg/ml) or AHBA (0.05~0.1 mg/ml) increased FR-900482 formation (data not shown). These results suggest a direct involvement of these compounds in FR-900482 biosynthesis. Then, we tried to clarify the mode of incorporation of D-glucosamine into FR-900482 (**1**) by the feeding experiment using D-[1-^{13}C]glucosamine.



1A



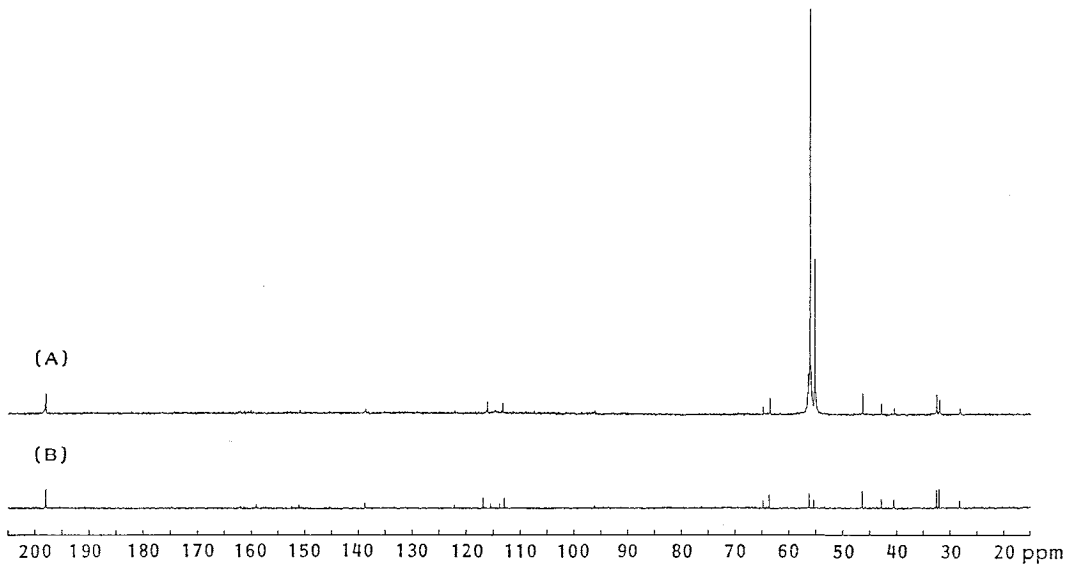
1B

Table 1. Incorporation of radioactively labeled precursors into FR-900482.

Compounds (specific activity mCi/mmol)	Original activity ($\times 10^6$ dpm)	FR-900482 production[a] (mg/10 ml)	Incorporation rate (%)
D-[*U*-^{14}C]Glucose (290)	15.5	1.45	0.7
D-[1-^{14}C]Glucose (59)	16.7	0.74	0.3
D-[3,4-^{14}C]Glucose (10.3)	14.2	1.10	0.7
D-[*U*-^{14}C]Glucosamine hydrochloride (309)	13.5	1.64	21.0
D-[1-^{14}C]Glucosamine hydrochloride (50)	16.8	1.11	12.0
[3-^{14}C]Pyruvic acid, sodium salt (18.5)	16.5	0.90	1.1
[2-^{14}C]Acetic acid, sodium salt (54.0)	15.0	1.08	0.2
[7-^{14}C]-3-Amino-5-hydroxybenzoic acid (26.4)	47.0	2.80	23.4

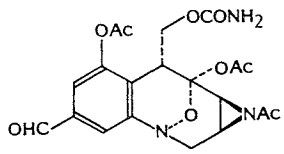
[a] The amount of FR-900482 produced in individual cultures was determined by the HPLC[1].

Fig. 1. ^{13}C NMR spectra of FR-900482 (100 MHz, D_2O, pD=7).
(A) ^{13}C-Enriched FR-900482 obtained by incorporation of D-[1-^{13}C]glucosamine. (B) Natural absorbance.



D-[1-^{13}C]Glucosamine (250 mg, 98.5 atom % ^{13}C) was added to fermentation broths (1 liter) at 72 hours. After 72 hours of additional incubation, the broths were centrifuged to remove the mycelia. The purification of ^{13}C-FR-900482 (90 mg) was accomplished according to the published procedure[1]. The 100 MHz ^{13}C NMR spectrum in D_2O exhibited marked enhancements of the signals at δ 56.2 and 55.3 as shown in Fig. 1. As reported in a previous paper[2], FR-900482 (**1**) is composed of two tautomers **A** and **B** (**A**:**B**=*ca.* 2:1 in D_2O). The enhanced signals correspond to the 13-carbons of **A** and **B**, respectively. This fact revealed that the 1-carbon of D-glucosamine was incorporated into the 13-position of **1**.

Since the ^{13}C NMR spectrum of ^{13}C-**1** itself was rather complicated due to the tautomeric mixture of **A** and **B**, ^{13}C-**1** was converted to ^{13}C-triacetate **2** so that it might be easy to analyze the NMR spectrum. Acetylation of ^{13}C-**1** with acetic anhydride in pyridine gave ^{13}C-**2** as a major product, the ^{1}H and ^{13}C NMR signals of which were completely assigned with the aid of the two-dimensional (2D) NMR technique[2]. The enhanced intensity of the ^{13}C satellite signals of the 12-carbon (δ 31.6, $J_{C12,C13}$= 43.5 Hz) was 26.0%. This value was in good agreement with the data (27.9%) obtained from the electron impact mass spectrometry (EI-MS) spectrum of ^{13}C-**2** in comparison with that of compound **2** derived from the cold FR-900482.



2

In conclusion, these biogenetic studies of FR-900482 indicate the possibility that AHBA is a possible precursor of FR-900482 and that D-glucosamine is incorporated as an intact unit into the antibiotic.

References

1) KIYOTO, S.; T. SHIBATA, M. YAMASHITA, T. KOMORI, M. OKUHARA, H. TERANO, M. KOHSAKA, H. AOKI & H. IMANAKA: A new antitumor antibiotic, FR-900482. II. Production, isolation, characterization and biological activity. J. Antibiotics 40: 594~599, 1987
2) UCHIDA, I.; S. TAKASE, H. KAYAKIRI, S. KIYOTO, M. HASHIMOTO, T. TADA, S. KODA & Y. MORIMOTO: Structure of FR-900482, a novel antitumor antibiotic from a *Streptomyces*. J. Am. Chem. Soc. 109: 4108~4109, 1987
3) BECKER, A. M.; A. J. HERLT, G. L. HILTON, J. J. KIBBY & R. W. RICKARDS: 3-Amino-5-hydroxybenzoic acid in antibiotic biosynthesis. VI. Directed biosynthesis studies with ansamycin antibiotics. J. Antibiotics 36: 1323~1328, 1983
4) GHISALBA, O. & J. NÜESCH: A genetic approach to the biosynthesis of the rifamycin-chromophore in *Nocardia mediterranei*. II. Isolation and characterization of a shikimate excreting auxotrophic mutant of *Nocardia mediterranei* with normal rifamycin-production. J. Antibiotics 31: 215~225, 1978
5) RINEHART, K. L., Jr.; M. POTGIETER, W. JIN, C. J. PEARCE, D. A. WRIGHT, J. L. C. WRIGHT, J. A. WALTER & A. G. MCINNES: Biosynthetic studies on antibiotics employing stable isotopes. *In* Trends in Antibiotic Research. Genetics, Biosyntheses, Actions & New Substances. *Ed.*, H. UMEZAWA *et al.*, pp. 171~184, Japan Antibiotics Res. Assoc., Tokyo, 1982
6) ANDERSON, M. G.; J. J. KIBBY, R. W. RICKARDS & J. M. ROTHSCHILD: Biosynthesis of the mitomycin antibiotics from 3-amino-5-hydroxybenzoic acid. J. Chem. Soc. Chem. Commun. 1980: 1277~1278, 1980
7) HORNEMANN, U. & M. AIKMAN: Mitomycin biosynthesis by *Streptomyces verticillatus*. Incorporation of the amino group of D-[^{15}N]-glucosamine into the aziridine ring of mitomycin B. J. Chem. Soc. Chem. Commun. 1973: 88~89, 1973