FK409, A NOVEL VASODILATOR ISOLATED FROM THE ACID-TREATED FERMENTATION BROTH OF *STREPTOMYCES GRISEOSPOREUS*

III. REACTION MECHANISM AND SYNTHESIS

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FK409 (**1**), a novel vasodilator, is a semi-artificial fermentation product of *Streptomyces griseosporeus* No. 16917, which was cultured in a medium containing nitrate. And the acid-treatment of the broth is essential for the generation of FK409.

FK409 was considered to be formed *via* a novel synchronous nitrosation-nitration reaction of FR-900411 (**2**) which was produced by the strain as a precursor of FK409. The conversion of FK-900411 to FK409 proceeded under acidic conditions with nitrite formed by microbial reduction of nitrate.

Total synthesis of FK409 was achieved starting from (*E*)-2-ethyl-2-butenal (**3**) *via* a nitrosation reaction of FR-900411 (**2**) as a key step.

FK409 (**1**) is a novel potent vasodilator isolated from the acid-treated fermentation broth of *Streptomyces griseosporeus* No. 16917[1]. The structural elucidation of FK409 (**1**) and its biosynthetic precursor FR-900411 (**2**), isolated from the fermentation broth of the producing strain has been reported[2]. This report describes the reaction mechanism of the chemical conversion of FR-900411 (**2**) to FK409 (**1**), and the total synthesis of FK409 (**1**) through FR-900411 (**2**).

Results and Discussion

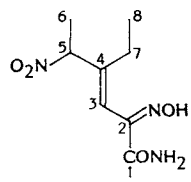
Generation of FK409 in the Fermentation Broth of *S. griseosporeus* No. 16917

The sequence of conversion of FR-900411 (**2**) in the broth to FK409 (**1**) is complex. In order to clarify the condition of FK409 generation, vasodilating activity of 3 types of samples were tested on the aorta preparation. Each sample was prepared as follows:

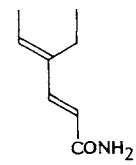
a: The fermentation broth cultured with the medium containing $NaNO_3$ was extracted with ethyl acetate after adjusting the pH to 3.0. Corresponding to 10 μl of the broth sample was applied to the assay. The medium composition was described previously[1].

b: The cultured broth was extracted without adjusting the pH to 3.0. Except the extraction, sample was prepared by the method of the same as **a**.

c: The fermentation was carried out with the medium omitted $NaNO_3$. Except the medium,

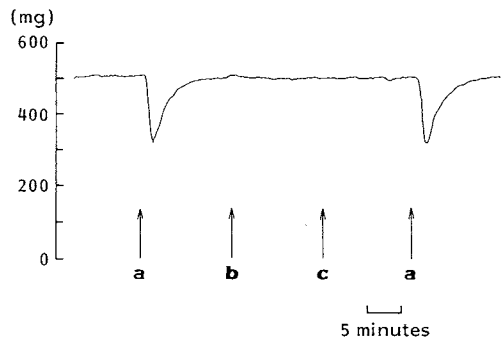


FK409 (**1**)



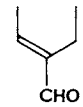
FR-900411 (**2**)

Fig. 1. Generation of FK409 in the fermentation broth.



Condition	Sample			
	a	b	c	a
$NaNO_3$ in the medium	+	+	−	+
Acid-treatment of the broth	+	−	+	+

The aorta was separated from a male SD rat weighing 250 g and superfused with Tyrode solution containing noradrenaline (0.03 μg/ml/minute) which increased the tension of the tissue by about 500 mg. The method was described previously[1].



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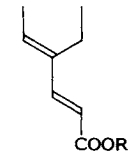

4 R=C_2H_5
5 R=H

same as **a**.

As shown in above, $NaNO_3$ in the medium and the acid-treatment of the fermentation broth are essential for the generation of FK409. From these observation, we found that FR-900411 produced in the broth reacted with $NaNO_2$ formed by microbial reduction of $NaNO_3$ under acidic condition.

Synthesis of FR-900411 (2)

In order to confirm the structure of FR-900411 (**2**), the compound was synthesized starting from (*E*)-2-ethyl-2-butenal (**3**) as follows.

Wittig reaction of (*E*)-2-ethyl-2-butenal (**3**) with triethyl phosphonoacetate in the presence of sodium hydride in benzene gave the diene-ester **4** which was hydrolyzed with aqueous 1 N NaOH in MeOH to yield the diene acid **5**. Treatment of this acid **5** with isopropyl chloroformate in the presence of triethylamine afforded the mixed anhydride which was converted into the amide **2** with ammonia gas. All spectral data of the synthetic amide **2** were superimposable on those of natural FR-900411[2], confirming *E*-configuration of the C-2–C-3 and C-4–C-5 double bonds in **2** and the whole structure of **2**.

Synthesis of FK409 (1)

We attempted to synthesize FK409 (**1**) by nitrosation reaction of FR-900411 (**2**). Treatment of the amide **2** with $NaNO_2$ at pH 3.0 (using 6 N HCl) gave the biologically-active compound **1**, identical in all respects with FK409, isolated from the acid-treated fermentation broth.

We infer the reaction mechanism for the transformation of FR-900411 (**2**) to FK409 (**1**) as follows. It is well known that $NaNO_2$ produces N_2O_3 complex gas in the presence of acid[3]. NO_2^- and NO^+ species might be formed *in situ* from N_2O_3 complex gas and then add to the conjugated diene system in the manner of a 1,4-dipole addition, so that NO_2^- attacks the carbon at the electron deficient position (C-5), while NO^+ attacks at the position of high electron density (C-2). From these results we confirm the structure of FK409 (**1**) and the reaction mechanism for the transformation of FR-900411 (**2**) to FK409 (**1**). To our knowledge, FK409 (**1**) is the first compound possessing the 1-nitro-4-oxime-2-butene system.

In the past, we have isolated many active compounds which were produced in the active form in the fermentation broth. Now though we have isolated FK409 from an acid-treated fermentation broth. Thus suggest the possibility of being able to discover new compound by chemical manipulation of the fermentation broth.

Experimental

IR spectra were recorded with a Jasco IRA-2 spectrometer. ^{1}H NMR spectra were measured on either a Jeol PMX-60 or a Jeol PS-100. The chemical shifts are given in ppm (δ) relative to an internal TMS standard, coupling constants (J) are in Hz and multiplicities are indicated by the usual symbols. UV spectra were measured on a Hitachi 220 A double beam spectrophotometer, absorption maxima are given in nm (extinction ε). Field desorption (FD)-MS was recorded using a Jeol JMS-D-300 mass spectrometer. MP's were measured with a Yanagimoto microscope hot-stage apparatus and are uncorrected.

Ethyl (2*E*,4*E*)-4-Ethyl-2,4-hexadienoate (**4**)

To a stirred suspension of sodium hydride (88 g, 60% in mineral oil) in anhydrous benzene (2,000 ml) was added dropwise triethyl phosphonoacetate (448 g) at 0°C under N_2. After the mixture had been stirred at room temperature for 30 minutes, (*E*)-2-ethyl-2-butenal (**3**) (196 g) was added dropwise at 0°C with stirring. The mixture was then stirred for additional 30 minutes at room temperature, diluted with water and extracted with EtOAc. The extract was washed with brine and dried over $MgSO_4$. Evaporation of the solvent gave an oily residue which was purified by distillation under reduced pressure to afford **4** (290 g): BP 120°C (20 mmHg); IR ($CHCl_3$) cm^{-1} 1690, 1620; ^{1}H NMR ($CDCl_3$) δ 7.20 (1H, d, J=16 Hz), 6.07~5.67 (2H, m), 4.20 (2H, q, J=7 Hz), 2.26 (2H, q, J=7 Hz), 1.78 (3H, d, J=7 Hz), 0.97 (3H, t, J=7 Hz); MS m/z 168 (M^+).

(2*E*,4*E*)-4-Ethyl-2,4-hexadienoic Acid (**5**)

A solution of **4** (134 g) and aqueous 1 N NaOH (880 ml) in MeOH (200 ml) was stirred at room temperature for 20 hours under N_2. Evaporation of the MeOH gave a residue which was taken up in water and washed with Et_2O. The aqueous solution was acidified with concd HCl and extracted with EtOAc. The extract was washed with brine and dried over $MgSO_4$. Evaporation of the solvent gave a crystalline material which was recrystallized from Et_2O to afford **5** (108 g): MP 79~81°C; IR ($CHCl_3$) cm^{-1} 1680, 1620; ^{1}H NMR ($CDCl_3$) δ 11.20 (1H, br s), 7.33 (1H, d, J=16 Hz), 6.00 (1H, q, J=7 Hz), 5.80 (1H, d, J=16 Hz), 2.29 (2H, q, J=7 Hz), 1.83 (3H, d, J=7 Hz), 1.00 (3H, t, J=7 Hz); MS m/z 140 (M^+).

(2*E*,4*E*)-4-Ethyl-2,4-hexadienecarboxamide (**2**)

To a stirred solution of **5** (70.0 g) and triethylamine (50.6 g) in CH_2Cl_2 (500 ml), isopropyl chloroformate (61.3 g) was added dropwise at −20°C and the mixture was stirred for 1 hour at the same temperature, after which time dry ammonia gas was bubbled into the mixture at −20°C for 1 hour. The reaction mixture was then washed with water, and the organic layer washed with aqueous 5% NaOH and brine, and dried over $MgSO_4$. Evaporation of the solvent gave a pale yellow oil, which was crystallized from Et_2O affording **2** (62 g) which was identical in all respects with FR-900411 obtained from natural sources: MP 57~58°C; UV λ_{max}^{MeOH} nm (ε) 260 (20,000); IR ($CHCl_3$) cm^{-1} 1670, 1620, 1590; ^{1}H NMR ($CDCl_3$) δ 7.17 (1H, d, J=16 Hz), 5.89 (1H, q, J=7 Hz), 5.83 (1H, d, J=16 Hz), 6.10~5.60 (2H, br s), 2.27 (2H, q, J=7 Hz), 1.78 (3H, d, J=7 Hz), 1.00 (3H, t, J=7 Hz); MS m/z 139 (M^+).

(3*E*)-4-Ethyl-2-hydroxyimino-5-nitro-3-hexenecarboxamide (**1**)

$NaNO_2$ (60 g) was added to a stirred solution of **2** (31.4 g) in 10% aqueous MeOH (1,500 ml). The pH of the mixture was adjusted to 3.0, and maintained by occasional addition of concd HCl, and monitoring with a pH meter. After 15 minutes, additional $NaNO_2$ was added to the solution and the mixture was kept at pH 3.0 with addition of concd HCl and the mixture was stirred at room temperature for 15 minutes. The reaction mixture was extracted with EtOAc, washed with brine, and dried over $MgSO_4$. Evaporation of the solvent gave a crystalline residue which was washed with hot $CHCl_3$, recrystallized from MeOH affording **1** (20 g) which was identical in all respects with FK409 obtained from natural sources: MP 140°C (dec); UV λ_{max}^{MeOH} nm (ε) 240 (sh, 7,000); IR (Nujol) cm^{-1} 3500, 3300~3200, 1660, 1600, 1560; ^{1}H NMR (acetone-d_6) δ 11.17 (1H, br s), 7.09 (1H, br s), 6.63 (1H, br s), 6.20 (1H, s), 5.37 (1H, q, J=7 Hz), 2.22 (2H, q, J=7 Hz), 1.72 (3H, d, J=7 Hz), 1.02 (3H, t, J=7 Hz); MS m/z 215 (M^+).

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