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A NEW NITRIC OXIDE (NO) RELEASER: SPONTANEOUS NO RELEASE FROM FK409

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The remarkable vasorelaxant and anti-platelet effects of FK409 have been reported to be due to nitric oxide (NO) release. The purpose of the present study is to investigate the spontaneous NO-releasing pathway of FK409 in aqueous solutions. ^{1}H-NMR spectra of FK409 suggested that the compound underwent a time-dependent elimination of the hydrogen atom at α-position of the nitro moiety (at the 5-position) in weakly alkaline solutions. In addition, the degradation of FK409 monitored by HPLC showed a pH-dependency accelerating with an increase of pH. These results revealed that the first step in the degradation of FK409 might be the hydroxyl ion-dependent subtraction of the hydrogen atom at the 5-position. On the other hand, NO release from FK409 also exhibited a pH-dependency, and the velocity of NO liberation was markedly enhanced above pH 6. Furthermore, a linear relationship between the rate of FK409 degradation and that of NO formation was observed, indicating that the rate-limiting step for NO formation is the same as that for degradation. Thus, the rate-limiting process of NO formation from FK409 is due to the deprotonation reaction of the hydrogen atom at the 5-position by hydroxyl ions. The deprotonation process appears to be an essential step for both FK409 degradation and NO release. On the basis of the results, a possible kinetic scheme for NO release from FK409 is proposed.

KEY WORDS: FK409, nitric oxide, degradation, hydroxyl ion.

INTRODUCTION

FK409, (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (Figure 1), isolated from the acid-treated fermentation broth of *streptomyces griseosporeus*, shows both potent vasorelaxant and anti-platelet activities.[1] The remarkable vasorelaxant effect of FK409 is due to the activation of soluble guanylate cyclase followed by an increase in cyclic guanosine-5′-monophosphate (cGMP) levels in isolated coronary artery rings of the dog[2] and rabbit arteries.[3] These effects are similar to the organic nitrates such as isosorbite dinitrate (ISDN) and glyceryl trinitrate (GTN).[4–7]

Recently, Kita *et al.* have found that both depression of mean blood pressure[8] and the cardioprotective effect[9] of FK409 are attributable to the elevation in plasma cGMP

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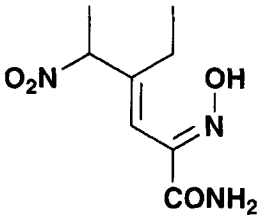

FIGURE 1 Chemical structure of FK409

levels in rats, and demonstrated that FK409 spontaneously releases nitric oxide (NO) in phosphate buffer at pH 7.4 as detected by chemiluminescence analysis.[8] Furthermore, they have reported that the anti-platelet effects on human platelets[10] and the antianginal effects[11] of FK409 are higher than those of ISDN, these effects being based on the ability of spontaneous NO generation. From these facts, the active pharmacological component of FK409 is suggested to be the NO molecule which has generally been accepted to be the endothelium-derived relaxing factor (EDRF).[12–14]

The development of pharmacological actions *via* NO in organic nitrates requires thiol compounds like cysteine,[13–15] in which NO production is thought to be initiated by the nucleophilic attack of thiolate anions on the nitrogen atom of their nitro ester groups.[16] In contrast to organic nitrates, the NO formation in FK409 is not due to the interaction with thiol-containing compounds. Since FK409 has no nitro ester group, another reaction mechanism leading to NO release in FK409 is suggested.

We investigated the formation of NO from FK409 with several physico-chemical techniques and found the hydroxyl ion-dependent NO release from the compound. This paper reports the results, proposing a possible kinetic scheme for the NO release from FK409 in the presence of hydroxyl ions.

MATERIALS AND METHODS

Chemicals

FK409 was synthesized at Fujisawa Pharmaceutical Co., Ltd (Osaka, Japan). Sulfanic acid and N-(1-naphthyl)-ethylenediamine dihydrochloride were purchased from Nacalai Tesque (Kyoto, Japan). Carboxy-PTIO (2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) was obtained from Dojindo Laboratories (Kumamoto, Japan). NO gas with 99.0% purity was obtained from Sumitomo Seika Chemicals (Osaka, Japan). To prepare the NO-saturated solution (ca.1.9 mM at 25°C), NO gas was bubbled into deionized water, which was deoxygenated with argon. The buffer systems used were as follows: 0.1 M HCl-NaCl, pH 1,2; 0.1 M sodium phosphate-NaCl, pH 4, 6, 7, 8; and 0.1 M Na_2CO_3-$NaHCO_3$-NaCl, pH 10. In each buffer system, the ionic strength was adjusted to 0.3.

Degradation-profile of FK409 Estimated by HPLC

FK409 (final concentration 1.5 mM) was added to the buffer solutions at pH 2 to 10. FK409 was dissolved in each buffer solution, and the solutions were incubated at 37°C. After appropriate reaction times, 2 ml from the sample solutions was taken and mixed with 2 ml of 2% (V/V) trifluoroacetic acid solution to stop the degradation of FK409.

Concentration of FK409 was determined by injecting 5 μl of the sample solution into HPLC system, which consisted of a SPD-2A variable-wavelength detector (Shimadzu, Kyoto, Japan) operating at 230 nm; a LC-9A pump (Shimadzu); a SIL-9A auto injector (Shimadzu) and a C-R3A integrator (Shimadzu) for peak processing.

The HPLC conditions were as follows: column, a YMC-Pack ODS-AM (4.6 mm i.d. × 15 cm, YMC, Kyoto, Japan); mobile phase, water:acetonitrile:trifluoroacetic acid (1500:500:1); flow rate, 1.0 ml/min.

FK409 Degradation Estimated by ^{1}H–NMR Spectroscopy

FK409 was dissolved in 0.1 M phosphate buffer solutions at pD 7.8 (dissolved in D_2O), and at appropriate time intervals, ^{1}H–NMR spectra were recorded with a model AC 200P (200 MHz, Bruker, Karlsruhe, Germany) at 37°C. Tetramethylsilane (TMS) was used as an internal standard of the chemical shift.

Determination of Concentration of NO Released from FK409

Concentration of NO generated from FK409 was measured by X-band ESR spectrometer, JES-RE3X (JEOL, Tokyo, Japan) using carboxy-PTIO.[17] Conditions for ESR measurement were as follows: modulation frequency, 100 KHz; modulation amplitude, 0.05 mT; scanning field, 337.2 ± 5 mT; response time, 0.03 sec; sweep time, 2 min; microwave power, 4 mW.

The sample solutions containing 0.1 mM carboxy-PTIO and 0.1 mM FK409 at pH 2 to 10, were incubated at 37°C. After appropriate time intervals, 20 μl of the solution was transformed rapidly into a capillary glass tube in an ESR cavity, and the ESR spectrum was recorded. Since NO detection by carboxy-PTIO may be influenced by alkaline pH value, the ESR spectrum at pH 10 was also recorded after re-adjusting the pH to a neutral value [0.5 ml of the sample solution was mixed with 10 μl of dilute phosphoric acid (20%, V/V), and the pH of the solution was adjusted at about 6.7].

Carboxy-PTIO has been reported to react with NO in a mole ratio of 1:1,[17] hence, the concentration of NO released from FK409 was calculated from the decrease of peak heights at the lowest magnetic field of the ESR signals due to carboxy-PTIO using manganese oxide as an internal standard. A calibration curve for determining the NO concentration was obtained with various amounts of NO-saturated solution in the presence of 0.1 mM carboxy-PTIO solution, confirming that carboxy-PTIO reacts with NO in a mole ratio of 1:1. The linear regression (r) was found to be 0.994.

Determination of Nitrite Concentration

To determine the nitrite concentration released, FK409 (1.5 mM) was dissolved in buffer solutions at pH 2 to 10. The solutions were incubated at 37°C. After appropriate reaction times, 0.05 ml of the sample solution was removed, and it was treated with 3.95 ml of 0.5 M HCl, 0.5 ml of 0.2% (W/V) sulfanic acid, and subsequently 0.5 ml of 0.1% (W/V) N-(1-naphthyl)-ethylenediamine dihydrochloride.[18] Absorbance at 548 nm due to a purple dye was measured by a UV-2200 spectrophotometer (Shimadzu). A standard curve was obtained with sodium nitrite under the same conditions.

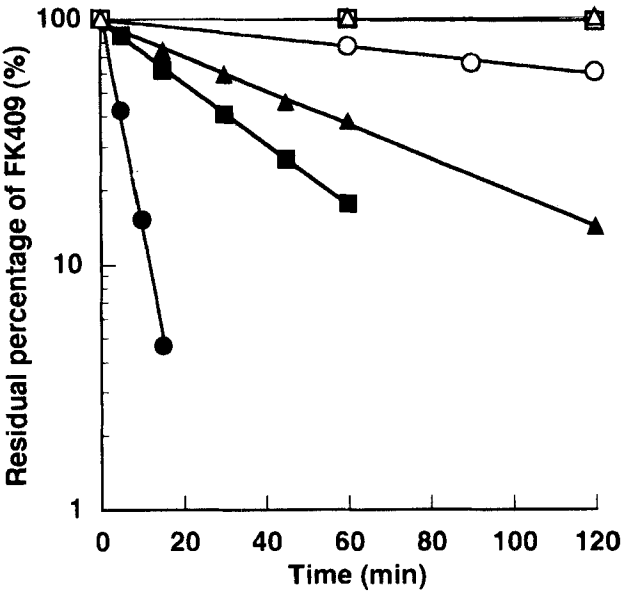

FIGURE 2 Degradation profile of FK409 in buffer solutions at pH 2 to 10 [pH 2 (△), pH 4 (□), pH 6 (○), pH 7 (▲), pH 8 (■), pH 10 (●)] at 37°C. The initial concentration of FK409 was 1.5 mM.

RESULTS

Degradation-profile of FK409

The degradation-profile of FK409 was studied in aqueous solutions at 37°C at pH 2 to 10. The pH-dependent degradation of FK409 is shown in Figure 2. Pseudo-first order kinetics were observed at all pH values, in which the degradation rate constants at each pH value were obtained from linear least-squares regression of semilogarithmic first-order plots. As summarized in Table 1, FK409 was found to be stable at low pH and unstable at high pH.

In order to understand the degradation mechanism of FK409, we used ^{1}H-NMR spectroscopy. The time-dependent changes of ^{1}H-NMR spectra for FK409 in buffer solutions at pH 1 to 8 were studied. As a typical example, the ^{1}H-NMR spectra in 0.1 M phosphate buffer solution (at pD 7.8) at initial and 30 min are shown in Figure 3. The signals of FK409 were assigned as follows: triplet at 0.99 ppm [3H, (a)] and quartet at 2.14 ppm [2H, (c)] from $CH_3CH_2^-$ doublet at 1.76 ppm [3H, (b)] and quartet at 5.45 ppm

TABLE 1
Degradation rate constants of FK409

	pH2	pH4	pH6	pH7	pH8	pH10
rate constant (min^{-1})	9.28×10^{-5}	2.29×10^{-4}	3.78×10^{-3}	1.64×10^{-2}	2.93×10^{-2}	1.97×10^{-1}
correlation coefficient	0.9993	0.9987	0.9988	0.9995	0.9997	0.9993

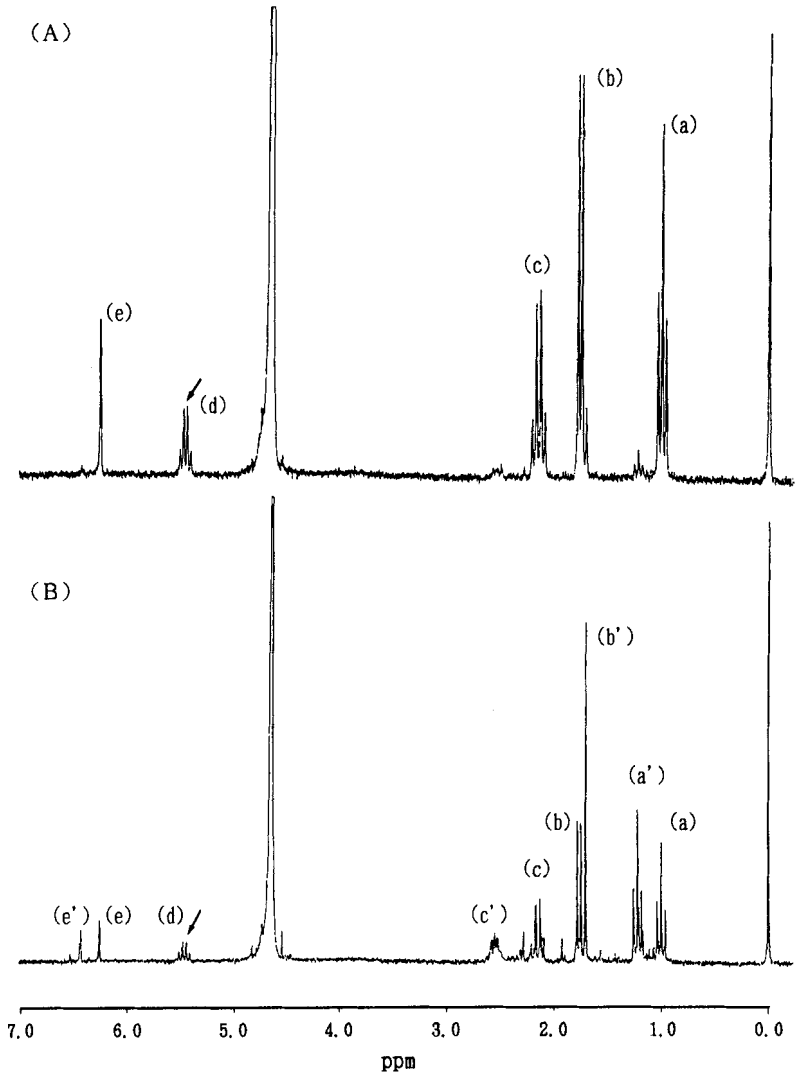

FIGURE 3 Time-dependent ^{1}H-NMR spectral change of FK409 in 0.1 M phosphate buffer solution (pD 7.8) at 37°C. (A) and (B) indicate the initial NMR spectrum and that after 30 min standing, respectively. The initial concentration of FK409 was 7.5 mM.

[1H, (d)] from $CH_3CH(NO_2)$-; singlet at 6.25 ppm [1H, (e)] from –CH=C<.

After 30 min, the signals due to the degradation products of FK409 have appeared concomitantly with the elimination of the signals of FK409. When FK409 degraded, the signals, except for that at 5.45 ppm, shifted as shown in Figure 3(B); (a) → (a′), (b) → (b′), (c) → (c′), (d) → no signal, (e) → (e′). The signal at 5.45 ppm (d) had gradually decreased, and disappeared after 90 min. These results indicate that the hydrogen atom

at the 5-position is extracted as the pH of the solution increases, suggesting the contribution of a hydroxyl ion-dependent degradation process of FK409.

pH-Dependent NO Formation from FK409

Akaike *et al.* previously described the antagonistic effect of carboxy-PTIO against NO by a bioassay technique.[17] They indicated that carboxy-PTIO reacts with authentic NO, and subsequently carboxy-PTI is generated by the reaction, the mole ratio being 1:1 (mol/mol) as follows.[17]

$$\text{carboxy-PTIO} + \text{NO} \rightarrow \text{carboxy-PTI} + \text{NO}_2$$

In addition, NO detection with carboxy-PTIO was found not to be affected by nitrite and nitrate (up to 10 mM).[19] Since the signal due to carboxy-PTIO at the lowest magnetic field is not overlapped by the signals of carboxy-PTI, the NO concentration released from FK409 can be determined by the peak height due to the signal with a calibration curve.

The time-dependent formation of NO release from FK409 is shown in Figure 4. Although the rate of NO liberation from FK409 increased from pH 2 to 8, the rate at pH 10 was smaller than that at pH 8. These results indicate that either NO is trapped slowly by carboxy-PTIO under alkaline conditions or NO is formed from an unknown intermediate during the degradation of FK409. The NO trapping ability of carboxy-PTIO at pH 10 is the same as that at neutral pH, based on the experimental results using NO gas. When the NO detection at pH 10 was carried out after adjustment at

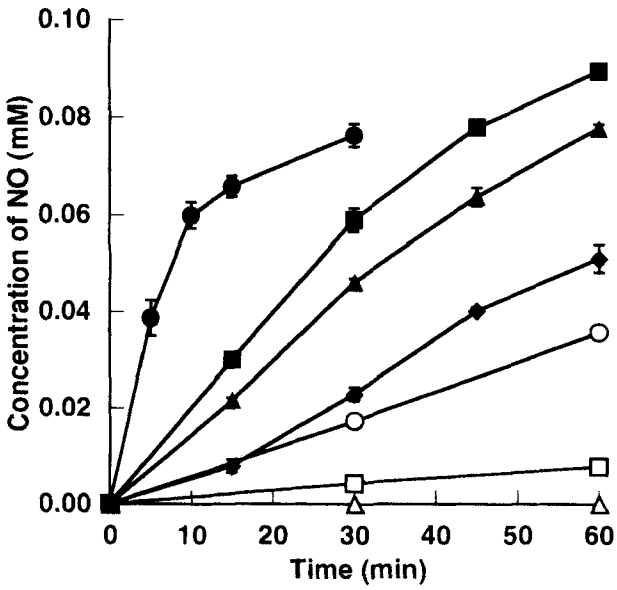

FIGURE 4 Spontaneous NO release from FK409 (0.1 mM) in buffer solutions at pH 2 to 10 [pH 2 (△), pH 4 (□), pH 6 (○), pH 7 (▲), pH 8 (■), pH 10 (adjusted at neutral pH, ●), pH 10 (no treatment, ♦)] at 37°C. NO was monitored with ESR spectral change due to carboxy-PTIO (0.1 mM). The results represent the means ± SEMs for three experiments.

TABLE 2
Rate constants for NO formation from FK409

	pH6	pH7	pH8	pH10
rate constant (min^{-1})	0.00766 ± 0.00026	0.0223 ± 0.0004	0.0308 ± 0.0012	0.145 ± 0.007
correlation coefficient	0.9970 ± 0.0007	0.9953 ± 0.0007	0.9939 ± 0.0009	0.9953 ± 0.0007

The results represent the means ± SEMs for three experiments.

neutral pH, the NO formation was found to be enhanced in a pH-dependent fashion (Figure 4). From these results, NO is assumed to be generated from the NO intermediate formed during FK409 degradation. The intermediate may decompose slowly to NO under alkaline solution, and decompose rapidly to NO under neutral one.

On the assumption that the NO generation follows first-order kinetics, the rate constants were calculated from the plots for the ([NO]∞ – [NO]) as a function of time at pH 6 to 10. The rate constants calculated are summarized in Table 2.

pH-Dependent Nitrite Generation from FK409

It is known that NO reacts with di-oxygen to produce nitrogen dioxide, which in turn forms nitrite and nitrate in aqueous solution under aerobic conditions.[12] Thus, we examined nitrite generation from FK409. The time-dependent nitrite production from FK409 is shown in Figure 5, in which first-order generation of nitrite was observed. At pH 10, the rapid generation of nitrite was observed.

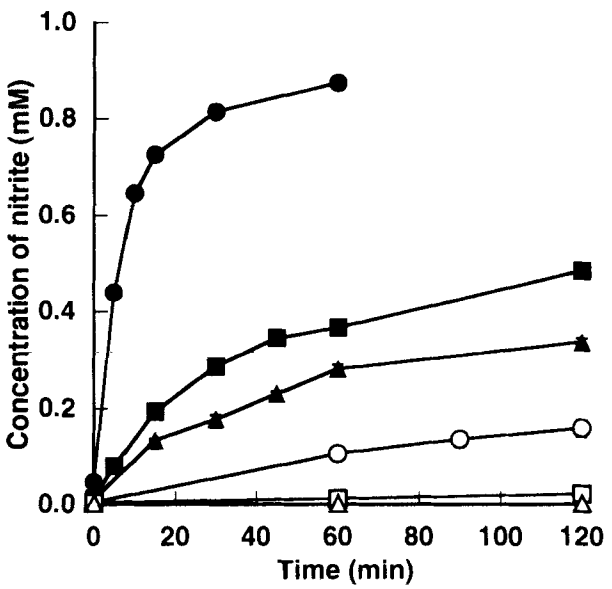

FIGURE 5 Time-dependent nitrite generation from FK409 in buffer solutions at pH 2 to 10 [pH 2 (△), pH 4 (□), pH 6 (○), pH 7 (▲), pH 8 (■), pH 10 (●)] at 37°C. The initial concentration of FK409 was 1.5 mM. The results represent the means ± SEMs for three experiments.

TABLE 3
Rate constants for nitrite generation from FK409

	pH6	pH7	pH8	pH10
rate constant (min^{-1})	0.00411 ± 0.00020	0.0102 ± 0.0004	0.0190 ± 0.0003	0.0983 ± 0.0084
correlation coefficient	0.9993 ± 0.0002	0.9849 ± 0.0011	0.9953 ± 0.0003	0.9769 ± 0.0166

The results represent the means ± SEMs for three experiments.

The rate constants calculated from the plots for the ([nitrite]$_\infty$ − [nitrite]) as a function of time in each buffer solution at pH 6 to 10, indicated that the nitrite generation depends on the pH values of the solution similarly to the NO generation (Figure 4). The calculated rate constants are summarized in Table 3.

DISCUSSION

The NMR measurements clearly showed that the acidity of the hydrogen atom at the 5-position plays an important role in the initiation of FK409 degradation (Figure 3). The nitro group, having an electron-withdrawing property increases the acidity of the 5-hydrogen atom, and enhances the stability of the carbanion formed. The high acidity of the 5-hydrogen atom facilitates its removal in the presence of a base such as hydroxyl ions. Therefore, at higher pH, the hydroxyl ion should accelerate the degradation of FK409. As expected, the degradation exhibited a pH-dependency and was markedly accelerated at high pH (Figure 2). The proton-abstraction should be the first step of FK409 degradation.

pH-Dependent NO formation from FK409 was observed (Figure 4), and the rate of NO liberation was strongly influenced by a change of pH: shifting the pH from 4 to 8 markedly enhanced the reaction rate of NO generation. On the other hand, NO formation was influenced under more extreme alkaline conditions, although the production of nitrite became faster with the increase of pH, as seen in Figure 5. Since carboxy-PTIO does not react with nitrite, NO appears to be generated from an intermediate formed by the degradation of FK409. The conversion of the intermediate to NO under alkaline conditions may be slower than under neutral ones. Probably, the intermediate is rapidly converted to nitrite under acidic conditions, because the nitrite was determined in 0.5 M HCl solution.

It is assumed that there is a difference in generation rate of NO and nitrite from FK409. Since nitrite is known to be generated by an oxidation of NO,[12] the rate of nitrite generation is related to both rate of NO formation from FK409 and that of NO conversion into nitrite. Therefore, the rate of nitrite generation is supposed to be a little smaller than that of NO formation.

As shown in Figure 6, a linear relationship between the rate of FK409 degradation and that of NO formation ($r = 0.9990$) was observed. NO is assumed to be generated several stages after FK409 degradation. However, the correlation between the rate of FK409 degradation and that of NO formation indicates that the rate-limiting process of NO formation is similar to that of FK409 degradation. In addition, we confirmed that there was also a linear relationship between the rate of FK409 degradation and that of nitrite production ($r = 0.9996$).

From these results, a possible kinetic scheme for NO generation from FK409 is

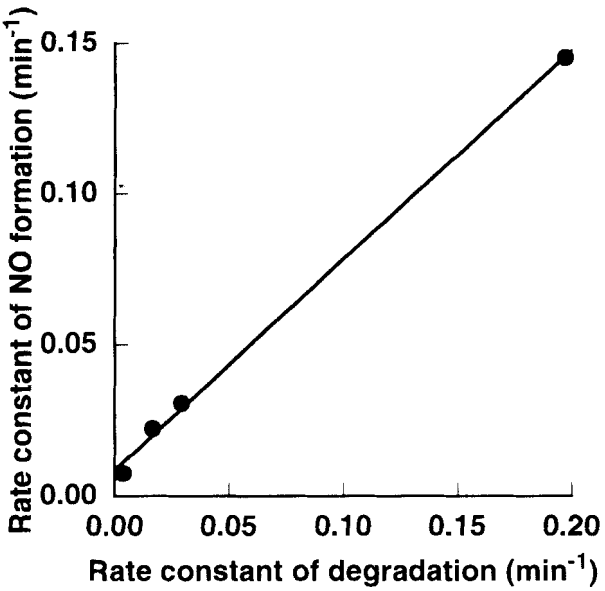

FIGURE 6 Relationship between the rate of FK409 degradation and that of NO formation from FK409

proposed in Scheme 1, in which the rate-limiting step of NO formation is the deprotonation of the hydrogen atom at the 5-position. This process is an essential step in the NO release from FK409. The rate of the NO release may be controlled by the basicity of the base used for the deprotonation reaction.

In conclusion, on the basis of the NO release from FK409, we propose here that FK409 is a new NO releaser which has the possibility to generate NO at specific sites, or under particular physiological conditions. FK409 should be a useful compound for more detailed elaboration of the physiological role of NO.

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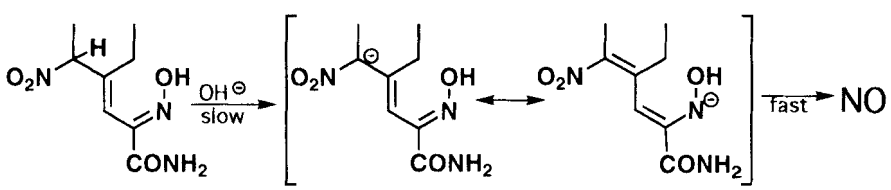

SCHEME 1 Proposed kinetic scheme of NO release from FK409

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