Pergamon

*Bioorganic & Medicinal Chemistry Letters*, Vol. 5, No. 9, pp. 973-978, 1995
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0960-894X/95 $9.50+0.00

0960-894X(95)00148-4

# DECOMPOSITION OF FK 409, A NEW VASODILATOR : IDENTIFICATION OF NITRIC OXIDE AS A METABOLITE

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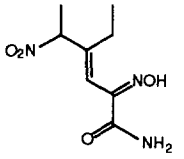
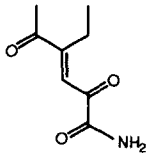
**Abstract**: (±)-(3E)-4-Ethyl-2-hydroxyimino-5-nitro-3-hexenamide, FK 409, is a natural product selected for its strong vasorelaxant properties. In neutral aqueous solution, it decomposes spontaneously giving rise to a diketone as shown by HPLC and mass spectrometry. Also, during decomposition, nitric oxide intermediate formation was demonstrated by EPR[1] spectroscopy specific assays. An analog of FK 409, in which the oxime group was selectively hydrolyzed, has lost the ability to generate NO and shows reduced vasorelaxant potency. These results provide a molecular basis for the vasorelaxant effects of FK 409.

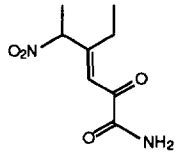
(±)-(3E)-4-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide, FK 409, is a new type of vasodilator isolated from the fermentation broth of *Streptomyces grisesporeus* as a semifermentation product.[2] FK 409 shows strong vasorelaxant effects and inhibits platelet aggregation.[3,4] These effects are associated with an increase in cyclic GMP content of the tissue due to the activation of soluble guanylate cyclase.[3] Moreover the vasorelaxant effect of FK 409 is independent of the integrity of the endothelium and is inhibited by oxyhaemoglobin, a nitric oxide scavenger. All these characteristics are shared by organic nitrates whose vasorelaxant properties depend on their activation to nitric oxide, NO.[5] It is now well established that NO is a potent activator of guanylate cyclase, a prerequisite for vasorelaxation. In order to understand the molecular mechanism by which FK 409 behaves as a vasodilator, we questioned whether the drug is also transformed to NO. In this paper, we demonstrate that FK 409 spontaneously decompose into **1** and NO in aqueous buffers, at room temperature. The direct evidence for NO formation was based on two EPR spectroscopy assays. During the preparation of this manuscript another study has also given some indication that NO can be produced from spontaneous FK 409 decomposition.[6]



**FK 409**



**1**



**2**

**Decomposition of FK 409.** The spontaneous decomposition of FK 409 at 24°C in aerated aqueous buffers was monitored by HPLC.[7] Initial reaction rates could be determined from the disappearence of the peak corresponding to FK 409 (Figure 1). Light and molecular oxygen were not involved in the reaction.
The decomposition was pH dependent with the highest rates observed at alkaline pH. At pH 7.5, about 83 % of the parent compound was lost, after 180 min, while it was still intact when the reaction was carried out at pH 6.0. FK 409 was stable at acidic pH (up to 4.5).

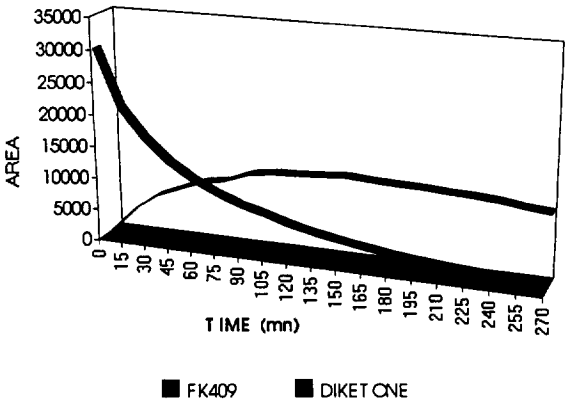



**Figure 1:** Respective peak areas of FK 409 and diketone 1 observed by LC/UV[7] (230 nm absorbance), pH=7.5.

At the end of the reaction the solution was yellow and the organic material was recovered essentially in one major peak and could be isolated. By mass spectrometry, the product was identified as the diketone **1**. The low resolution LC/MS[8] showed a pseudomolecular ion $(M+H)^+$ at m/z: 170 a.m.u.. The accurate mass measurement was in agreement with an elemental composition of $C_8H_{12}NO_3$. The Daughters of this ion observed by LC/MS/MS experiments were characteristic of the structure (Figure 2).

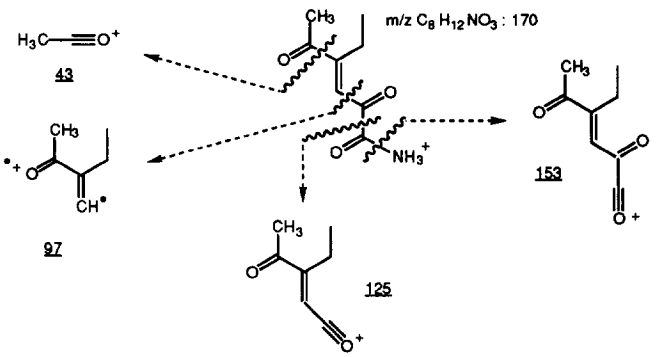



**Figure 2:** Fragmentation of pseudomolecular ion, m/z = 170, $(M+H)^+$ of diketone **1**.

As shown in Figure 3, during decomposition, FK 409 produced large amounts of nitrite $NO_2^-$.[9] The initial rate of $NO_2^-$ production as well as the total yield of $NO_2^-$ were pH dependent. For example, at pH 11, the overall yield was 65 % (based on the starting FK 409) and was obtained after 30 min incubation, while at pH

7.5, nitrite yield was only 13 % after 30 min and levelled off after 3 hours at about 30 %. Addition of iron chelators, such as desferrioxamine, or iron (II) salts did not affect the rates of $NO_2^-$ production.

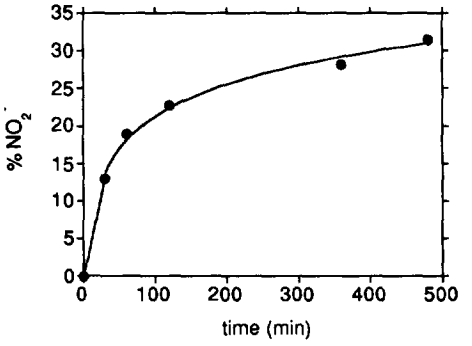



**Figure 3**: Nitrite formation during FK 409 decomposition.[9]

We also assayed $NO_2^-$ production during decomposition of compound **2**, an analog of FK 409 in which the oxime group was changed to a keto group by selective hydrolysis. This compound was a very poor vasodilator[10] ( $IC_{50}$= $7x10^{-6}$ M), when compared to FK 409 ( $IC_{50}$= $1.5x10^{-7}$ M). However, the propensity to produce nitrite spontaneously was even greater than that of FK 409. For example 37 % nitrite was measured after 30 min incubation at pH 7.5. There is thus no correlation between $NO_2^-$ production and the vasorelaxant properties, in the case of compound **2**.

**NO release from FK 409.** In order to demonstrate that NO was an intermediate product during the decomposition of FK 409 into nitrite, we used two scavengers of NO and EPR spectroscopy to monitor the scavenging reaction. The first NO trap was the $Fe^{2+}$– diethyldithiocarbamate (DETC) complex, dissolved in yeast cell membranes at pH 7.5.[11] After addition of FK 409, the EPR signal shown in Figure 4 was recorded. This signal with a triplet hyperfine structure at $g$ = 2.03 was characteristic of a paramagnetic mononitrosyl-$Fe^{2+}$–$(DETC)_2$ stable complex, resulting from the binding of NO to the $Fe^{2+}$–$(DETC)_2$ complex.[11] On the other hand, no nitrosyl-iron complex was formed when $Fe^{2+}$–$(DETC)_2$ was incubated with solutions of nitrite. When the same assay was applied to the decomposition of compound **2**, no EPR signal could be detected. This shows that nitrite ions derived from **2** were not produced through oxidation of NO.

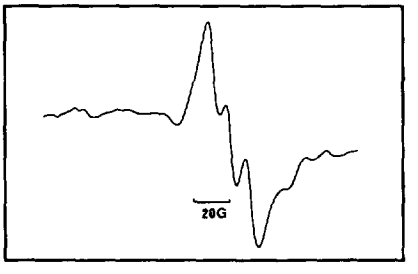
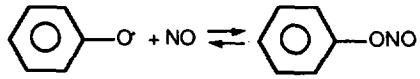



**Figure 4**: EPR spectrum of the paramagnetic NO-$Fe^{2+}$-$(DETC)_2$ complex during decomposition of 0.5 mM FK 409 in aqueous suspensions of yeast cells loaded with DETC.[11]

The second assay is based on the ability of NO to reversibly scavenge the tyrosyl radical of the small subunit of ribonucleotide reductase named protein R2 (Scheme 1).[12] This reactivity is an intrinsic property of phenoxyl radicals in general (Scheme 1).[13] Such a reaction is very specific for NO since $O_2$, $O_2^{\bullet -}$, $OH^{\bullet}$, $NO_2^-$, $NO_3^-$ are not able to carry out the same reaction.

*Scheme 1:*




A pure preparation of protein R2 displays an EPR signal at $g = 2.00$ characteristic of a stable tyrosyl radical. This signal disappears when protein R2 was incubated with FK 409 and later reappears (Figure 5), in agreement with the formation of NO from FK 409 first with the transient formation of EPR-silent nitrosotyrosine. Then NO reacts with molecular oxygen and its concentration decreases allowing the reformation of the tyrosyl radical. A small decrease of the intensity of the EPR signal during 2 h incubation was also observed in the absence of the drug just showing the slow spontaneous inactivation of the enzyme at 37°C. The tyrosyl radical was not affected by the presence of compound **2** confirming that compound **2** was not a NO donor.

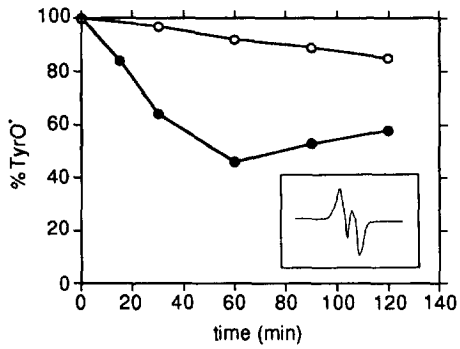



**Figure 5**: Loss of the tyrosyl radical EPR signal during incubation of 10 μM protein R2 with 1 mM FK 409 (●) in 0.1 M phosphate buffer pH 7.5 at 37°C.[12] A control experiment was carried out in the absence of FK 409 (○). The same curve was obtained during incubation of R2 with compound **2**. Inset: the EPR signal of the R2 tyrosyl radical recorded at 110 K.

In order to test whether oxygen radicals were formed during decomposition of FK 409, reaction mixtures containing FK 409 and DMPO as a spin trap for $O_2^{\bullet -}$ and $OH^{\bullet}$ radicals were analyzed by EPR spectroscopy at room temperature. However in no case could EPR signals be detected.

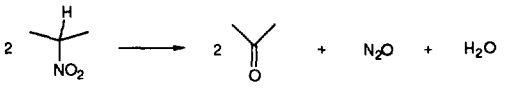
FK 409 has been recently discovered as a potent vasodilator. Its chemical structure differs from other types of vasorelaxant species such as organic nitrates, nitroprusside and S-nitrosothiols, whose pharmacological effects are related to their capacity to release NO. FK 409 does not contain any nitrate or nitroso group. However, it contains a nitro moiety as well as an oxime group that might be transformed into NO. In this study we show for the first time that FK 409 is actually a spontaneous NO donor when dissolved in aqueous

buffers. It is interesting to observe that removal of the oxime group completely inhibited NO formation and most of the vasorelaxant properties.

In general, the presence of nitrite is considered to be a good indicator for NO production since NO is immediately oxidised to nitrite. On the other hand, it is also believed that such an assay is not fully reliable. Here we report an example of a compound, **2**, which spontaneously generates nitrite with no intermediate formation of NO. Compound **2** thus could have been incorrectly characterized as a NO donor, which of course would have been in contradiction with its very poor vasorelaxant properties. This example clearly shows the importance of direct detection of NO for classification of a compound as a NO donor. FK 409 thus probably releases nitrite by different mechanisms, including oxidation of transient NO. The reason why only a small proportion of nitrogen atoms is recovered as nitrite is still not clear. A recent study using chemiluminescence detection has also shown that NO is released from FK 409 and that nitrate can be detected in the reaction mixture.[6] However, some other nitrogen metabolites still need to be identified.

It is a fascinating challenge to understand the molecular mechanism for the spontaneous decomposition of FK 409 into NO, a free radical species. At that stage, it would be too much speculative to make a proposal. However, such a mechanism would have to include the following features : (i) both the nitro and the oxime groups are needed for the generation of NO; (ii) both groups are hydrolyzed to the corresponding ketones; (iii) the reaction is accelerated at high pH. An attractive possibility is that FK 409 is the site of a Nef reaction (Scheme 2) even though it gives no indication for the origin of the radical chemistry. This would be consistent with the significant lability of the allylic proton in the α position with regard to the nitro group.

*Scheme 2:*




In Nef reactions, $NO^-$ is intermediately formed and transformed in $N_2O$.[14] $NO^-$, the nitroxyl ion, would be a likely precursor for NO. Further studies are needed to understand this mechanism.

During decomposition, FK 409 does not generate reduced oxygen species, as shown from spin-trapping experiments with DMPO. On the contrary, under similar conditions, the vasodilatory drug molsidomine, SIN-1, has been shown to autoxidize with production of both superoxide and NO, which together generate hydroxyl radicals.[15] One thus may expect FK 409 to be a less toxic vasodilator.

In conclusion, we have demonstrated that FK 409 decomposes in solution into the diketone **1** and nitrite with the intermediate formation of NO. The ability of NO to activate the soluble guanylate cyclase would then explain the vasorelaxant properties of the compound. This decomposition is spontaneous, as it is for S-nitroso thiols, and does not require redox activation contrary to organic nitrates.[6] Further studies are needed to understand this mechanism, in particular the search for $N_2O$, which would support the proposed Nef reaction and improve the nitrogen balance.

**Acknowledgements** We thank Dr P. Bellevergue for the preparation of compound **2** and Mrs Delahaye for performing the biological experiments and Dr S. E. O'Connor for helpful discussions and for criticism of an early version of this manuscript.

## References and notes


1. Abbreviations: DETC, diethyldithiocarbamate; EPR, Electron Paramagnetic Resonance; LC, Liquid Chromatography; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethane sulphonic acid]); MS, Mass Spectrometry; RNR, Ribonucleotide Reductase.
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7. LC/UV: A solution of FK 409 (0.1 M) was prepared in a phosphate buffer, pH 7.5. The degradation at 24 °C was monitored by LC/UV with an HP1090 instrument, working at 230 nM and coupled to an automatic injector. Separation was achieved on a Inertsil $C_{18}$ column (ALTECH: 25 cm x 4.5 mm x 5 µm) under isocratic conditions (phosphate buffer pH 7.5 / $CH_3CN$: 60 / 40, flow rate: 1 ml/min).
8. LC/MS: The diketone **1** was identified by LC/MS with a Plasmaspray interface on a hybrid sector MS/MS instrument (Autospec EQ FISONS). Accurate mass measurements were recorded at 6000 resolving power with a makeup of PEG 200, as a reference, just after the UV detector. The complete structure of the diketone was found by LC/MS/MS experiments with high and low energy collisions (MIKES and Daughters-Q).
9. Determination of nitrite (Bratton, A. C., Marshall, E. K. *J. Biol. Chem.* **1939**, *128*, 537-550): Nitrite concentrations were measured spectrophotometrically via diazotation of sulfanilamide and subsequent coupling with *N*-(1-naphthyl)ethylenediamine dihydrochloride to form the azo derivative.
10. Vasorelaxant properties: Isolated rabbit aorta strips were placed in organ bath solution, maintained at 37°C and bubbled with 95% $O_2$ and 5% $CO_2$ gas mixture. The resting tension was adjusted to 2 g and the strip was contracted with 3 µM of phenylephrine. Results are expressed as $IC_{50}$.
11. Detection of NO by paramagnetic NO-$Fe^{2+}$-$(DETC)_2$ complex (Mordvintcev, P., Mülsch, A., Busse, R., and Vanin, A. *Anal. Biochem.* **1991**, *199*, 142-146): a suspension of yeast cells (baker yeast bought in a local store; 200 mg/ml) in 0.1 M HEPES pH 7.5 was incubated with DETC (2.5 mg/ml) for 30 min at 37°C. The suspension (2 x 2 ml) was washed once by centrifugation and the plug were resuspended in 2 x 1 ml 0.1 M HEPES pH 7.5. To 200 µl of this suspension was added 200 µl of an aqueous solution containing FK 409 (0.5 mM). The reaction was performed during 15 min at 37°C. The samples were transferred into an EPR tube, frozen in liquid nitrogen and the EPR spectra were recorded at 110 K using a Varian E102 spectrometer.
12. Protein R2 was prepared from overproducing strains of *E.coli* (Larsson, A., Karlsson, M., Sahlin, M. and Sjöberg, B. M. *J. Biol. Chem.* **1988**, *263*, 17780-17784). Detection of NO with protein R2 (Roy, B., Lepoivre, M., Henry, Y., and Fontecave, M. *Biochemistry*, in press): the reaction was carried out at 37°C into an EPR tube containing protein R2 (1 mg/ml), FK409 (1 mM) in 200 µl of 0.1 M phosphate buffer pH 7.5. At time intervals, the tube was frozen in liquid nitrogen and the EPR spectrum of the solution was recorded at 110 K. The amount of tyrosyl radical was determined from the comparison of the amplitude of the typical EPR signal at $g$=2 to that of a pure sample of protein R2 (1 mg/ml).
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(*Received in Belgium* 8 *February* 1995; *accepted* 24 *March* 1995)