Communications to the Editor

STRUCTURE DETERMINATION OF THE PRIMARY RENAL METABOLITE OF THE PENEM FCE 22101

Sir:

The penem FCE 22101, sodium (5*R*,6*S*)-6-[(1*R*)-hydroxyethyl]-2-carbamoyloxymethyl-2-penem-3-carboxylate (**1**), is a new synthetic β-lactam antibiotic currently undergoing clinical evaluation. Promises of therapeutic usefulness are supported by its broad antimicrobial spectrum, the bactericidal mode of action and its resistance to a wide range of bacterial β-lactamases[1].

It has been reported that penem and carbapenem antibiotics are extensively metabolized[2] "*in vivo*" by a β-lactamase activity associated with the renal dipeptidase, dehydropeptidase I, located in the brush border membrane of mammalian kidneys[2~4]. Although after administration in mammals the urinary recovery of FCE 22101 was found to be higher[1,5] than that reported for the prototypic carbapenem imipenem (*N*-formimidoylthienamycin) and related carbapenems[3], it was mandatory to assess the stability of FCE 22101 to the renal dehydropeptidase and to elucidate the structure of the main product (**2**) resulting upon enzymic and hydrolytic cleavage.

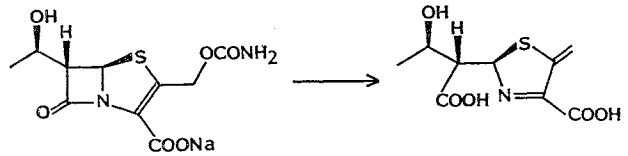
"*In Vitro*" Interaction Studies with Porcine Renal Dehydropeptidase I (DHP-I)

The enzyme DHP-I (E.C. 3.4.13.11)[2,3] was isolated from the cortex of fresh porcine kidney following the procedure described by CAMPBELL *et al.*[4]. The specific activity of purified DHP-I, homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis[4], was found to be about 50 μmol/minute/mg protein, using the standard 50 μM glycyldehydrophenylalanine assay at 37°C in 0.02 M Tris-HCl buffer at pH 8.0[6]. HPLC analyses were performed using a Hewlett-Packard 1090 A liquid chromatograph equipped with a Novapak C-18 column (4 μm, 150×3.9 mm i.d., Waters). Two mobile phases were utilized: A) 0.02 M KH_2PO_4, pH 2.5 (H_3PO_4) and B) phase A - CH_3CN (60:40). Gradient analyses were carried out at 1 ml/minute and 40°C, starting at 100% phase A and adding phase B at 2% B/minute from 0 up to 5 minutes and at 5% B/minute from 5 up to 12 minutes. FCE 22101 and compound **2** were eluted at about 8.7 and 7.3 minutes, respectively. Component identity and purity were confirmed by UV spectra obtained during the elution. Isocratic elution at 72:28 ratio phase A - phase B was adopted for kinetic studies to reduce the analysis time, FCE 22101 and compound **2** being eluted at 2.1 and 1.7 minutes, respectively. The DHP-I catalyzed hydrolysis of FCE 22101 was investigated at eight different initial substrate concentrations, over the range 0.1~27 mM, in aqueous 0.25 M 3-(*N*-morpholino)propanesulfonic

Table 1. Chemical stability of FCE 22101 over the 1.7~9.0 pH range[a].

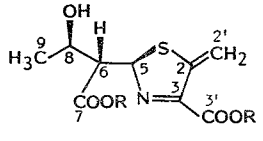
pH	Half life (hours)
1.7	4.6
2.7	24.5
3.7	91.4
4.4	163.0
5.5	172.0
6.4	131.0
7.0	105.0
8.0	30.8
8.5	16.8
9.0	5.85

[a] At 0.64 mM concentration in aqueous 0.1 M phosphate buffers at 37°C.

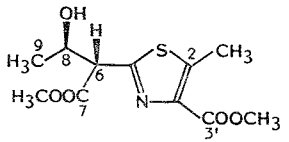


1 **2**

Table 2. Spectral data of the hydrolysis product of FCE 22101 and its derivatives.



2a R = Na
2b R = NH_4
3 R = CH_3



4

(A) IR, UV and field desorption mass spectra (FD-MS).

	IR (cm^{-1})[a]	UV (nm)[b]	FD-MS (*m/z*)
2b	1550~1620 (br)	298	—
3	1735, 1700	—	273
4	1735 (sh), 1715	245	273

[a] In $CHCl_3$ except **2b**, which was taken in KBr.
[b] In 95% EtOH.

(B) ^1H NMR (200 MHz, δ ppm).

		2′-H_2	2-CH_3	3′-OCH_3	5-H	6-H	7-OCH_3	8-H	9-H_3
2a	(D_2O, 45°C)	5.57, 5.61 (two dd, J=1.5, 1.5 Hz)	—	—	6.04 (ddd, J=1.5, 1.5, 7.5 Hz)	2.75 (dd, J=7.5, 8.5 Hz)	—	4.12 (dq, J=8.5, 6.3 Hz)	1.26 (d, J=6.3 Hz)
2b	(DMSO-d_6, 45°C)	5.29, 5.64 (two s)	—	—	5.78 (d, J=8.6 Hz)	2.36 (dd, J=7.0, 8.6 Hz)	—	3.84 (dq, J=6.0, 7.0 Hz)	1.07 (d, J=6.0 Hz)
3	($CDCl_3$)	5.60, 5.96 (two dd, J=1.8, 1.8 Hz)	—	3.91	6.26 (ddd, J=1.8, 1.8, 6.5 Hz)	3.00 (dd, J=6.5, 6.5 Hz)	3.71	4.30 (dq, J=6.5, 6.5 Hz)	1.30 (d, J=6.5 Hz)
4	($CDCl_3$)	—	2.76	3.91	—	4.18 (d, J=4.4 Hz)	3.74	4.52 (dq, J=4.4, 6.3 Hz)	1.23 (d, J=6.3 Hz)

(C) ^{13}C NMR (50.3 MHz, δ ppm).

	C_2	$C_{2'}$	C_3	$C_{3'}$	C_5	C_6	C_7	C_8	C_9
2b (D_2O, 25°C)	146.37	107.09	ns	ns	67.85	81.24	ns	63.34	21.08

ns: Not seen.

acid (MOPS) - NaOH buffer, pH 7.1, at 37°C in the presence of 2 mg/liter DHP-I.

For each run decreasing FCE 22101 concentrations were measured by HPLC injecting aliquots of the same solution every 3.8 minutes. From three to ten data points were acquired to define the early stage of each reaction. The initial degradation velocity was calculated extrapolating the slope of the plot of FCE 22101 concentrations against time to the "zero time". Fitting initial velocities and substrate concentrations to the Michaelis-Menten equation the following kinetic parameters were obtained:

Km=2.7 mM and Vmax=0.15 mM/minute/mg.

Chemical Stability Studies

The data reported in Table 1 indicate that FCE 22101 displays a good stability over a wide range of pH values in different buffered media including those selected for the "*in vitro*" interaction studies with porcine renal dehydropeptidase.

In neutral or alkaline aqueous media one major degradation product was formed; on the basis of the HPLC retention time and UV spectrum it proved to be identical to product **2** resulting from the DHP-I inactivation of FCE 22101.

Structural Elucidation of the Hydrolysis Product **2**

Early attempts to isolate compound **2** after enzymic hydrolysis failed, mainly owing to its chemical lability. Therefore, FCE 22101 was hydrolyzed in D_2O and the mixture analyzed by ^1H NMR spectroscopy. Typically, 5.4 mg of substrate in 50 ml D_2O was treated with purified DHP-I (0.076 mg as total protein content) at 37°C while keeping the pD of the solution at about 7.4 by the addition of 0.01 M NaOD in D_2O. After 4 hours an almost complete transformation to compound **2a** was confirmed by HPLC analysis and the solution was concentrated up to 0.4 ml. The recorded ^1H NMR spectrum (Table 2), in particular the signals at 5.57 and 5.61 ppm coupled (J=1.5 Hz) with the signal at 6.04 ppm, was strongly suggestive of an exomethylenethiazoline structure. Eventually, a sample of the diammonium salt **2b** was isolated upon chemical hydrolysis of FCE 22101 (1 g) in 0.05 M aqueous NaOH (250 ml) at 5°C overnight. The reaction mixture was brought to pH 8 by adding carboxylic exchange resin (Amberlite CG-50, H^+ form) and the filtrate was chromatographed on a Sephadex DEAE-A25 column (HCO_3^- form). After elution with aqueous 2% (w/v) NH_4HCO_3, selected fractions were concentrated and **2b** (260 mg) was precipitated with acetone. The UV, IR, ^1H and ^{13}C NMR spectra of this sample are reported in Table 2. The superimposition of the ^1H NMR spectra in D_2O of **2a** and **2b**, taken either separately or in admixture, confirmed their common structure. The exomethylenethiazoline structural assignment was corroborated by the conversion of **2a** and **2b** into the methylthiazole **4**, *via* esterification to **3** (CH_3I, DMSO - H_2O, 15 minutes, 25°C) followed by base-induced aromatization (NEt_3).

In conclusion, the present work demonstrates that one major product, the exomethylenethiazoline **2**, is formed either by chemical or *in vitro* enzymic hydrolysis of the penem FCE 22101. This result is reinforced by the observation that compound **2** was also found identical (HPLC, UV) to the major metabolite of FCE 22101 detected in the urine of different mammalian species, man included. Most likely, and in close analogy with the exomethylenedihydrothiazine or -oxazine resulting from the hydrolysis of cephalosporins[7] and oxacephems[8] carrying 3′-leaving groups, compound **2** also portrays the structure of the acyl portion of the acyl-enzyme intermediate resulting from the interaction of FCE 22101 and related penems with their bacterial targets (penicillin-binding proteins).

Compound **2** is structurally different from the carbapenem[9] and penem[10] hydrolysis products reported hitherto. Studies are in progress to define the possible biological implications of this finding.

Acknowledgment

We are grateful to E. ARLANDINI for mass spectra and to Istituto Mobiliare Italiano (IMI) for financial support.

GIUSEPPE CASSINELLI
RICCARDO CORIGLI
PIERGIUSEPPE OREZZI
GIAMBATTISTA VENTRELLA
ANGELO BEDESCHI
ETTORE PERRONE
DANIELA BORGHI
GIOVANNI FRANCESCHI

Farmitalia Carlo Erba S.p.A.,
Ricerca & Sviluppo,
Via dei Gracchi 35, 20146 Milan, Italy

(Received October 27, 1987)

References

1) FRANCESCHI, G.; M. FOGLIO, M. ALPEGIANI, C. BATTISTINI, A. BEDESCHI, E. PERRONE, F. ZARINI, F. ARCAMONE, C. DELLA BRUNA & A. SANFILIPPO: Synthesis and biological properties of sodium (5*R*,6*S*,8*R*)-6α-hydroxyethyl-2-carbamoyloxymethyl-2-penem-3-carboxylate (FCE 22101) and its orally absorbed esters FCE 22553 and FCE 22891. J. Antibiotics 36: 938~941, 1983

2) KIM, H. S. & B. J. CAMPBELL: β-Lactamase activity of renal depeptidase against *N*-Formimidoyl-thienamycin. Biochem. Biophys. Res. Commun. 108: 1638~1642, 1982

3) KROPP, H.; J. G. SUNDELOF, R. HAJDU & F. M. KAHAN: Metabolism of thienamycin and related carbapenem antibiotics by the renal dipeptidase, dehydropeptidase-I. Antimicrob. Agents Chemother. 22: 62~70, 1982

4) CAMPBELL, B. J.; L. J. FORRESTER, W. L. ZAHLER & M. BURKS: β-Lactamase activity of purified and partially characterized human renal dipeptidase. J. Biol. Chem. 259: 14586~14590, 1984

5) DELLA BRUNA, C.; G. CARELLA, W. GROPPI, D. JABES & A. SANFILIPPO: Oral absorption in man of the penem FCE 22891 and antibacterial activity of the simulated plasma AUC. Program and Abstracts of the 24th Intersci. Conf. on Antimicrob. Agents Chemother., No. 594, p. 191, Washington, D.C., Oct. 8~10, 1984

6) RENE', A. M. & B. J. CAMPBELL: Amino acid composition and effect of pH on the kinetic parameters of renal dipeptidase. J. Biol. Chem. 244: 1445~1450, 1969

7) FARACI, W. S. & R. F. PRATT: Mechanism of inhibition of the PC1 β-lactamase of *Staphylococcus aureus* by cephalosporins: Importance of the 3'-leaving group. Biochemistry 24: 903~910, 1985

8) NISHIKAWA, J.; F. WATANABE, M. SHUDOU, Y. TERUI & M. NARISADA: ^{1}H NMR study of degradation mechanisms of oxacephem derivatives with various 3'-substituens in alkaline solution. J. Med. Chem. 30: 523~527, 1987

9) SHIBAMOTO, N.; T. YOSHIOKA, M. SAKAMOTO, Y. FUKAGAWA & T. ISHIKURA: Pharmacological studies on carbapenem antibiotics. III. Chemical structure of PS-5D III, the primary renal metabolite of PS-5. J. Antibiotics 35: 736~741, 1982

10) PFAENDLER, H.R.; J. GOSTELI, R.B. WOODWARD & G. RIHS: Structure, reactivity, and biological activity of strained bicyclic β-lactams. J. Am. Chem. Soc. 103: 4526~4531, 1981