6-(SUBSTITUTED METHYLENE)PENEMS, POTENT BROAD SPECTRUM INHIBITORS OF BACTERIAL β-LACTAMASE

IV. KIDNEY STABILITY, SERUM BINDING AND ADDITIONAL BIOLOGICAL EVALUATION OF RACEMIC DERIVATIVES

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Sodium (5*RS*)-*Z*-6-(substituted methylene)penem-3-carboxylates (**3**) are extremely potent inhibitors of bacterial β-lactamases, but some members of this group of compounds are highly bound to human serum, while others are readily degraded by renal dehydropeptidase I enzyme. Consequently, the stability of a variety of 6-(substituted methylene)penems (**3**) to human kidney homogenate, their binding to human serum and their activity in a mouse infection model was investigated at an early stage, and were instrumental in the selection of the 1,2,3-triazolylmethylene derivatives (*e.g.* **3k**) as a class of compounds worthy of further evaluation.

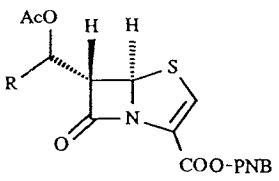
The *in vivo* activity of an antimicrobial agent is influenced by serum binding and metabolic stability. The enzyme renal dehydropeptidase I (RDHP) is known to be a major cause of metabolic inactivation of carbapenems such as imipenem, both in laboratory animals and man, and inhibition of this enzyme by the RDHP inhibitor cilastatin resulted in a 6- to 8-fold increase in urinary recovery and reduced nephrotoxic potential[1]. Certain penem antibiotics are also known to be hydrolysed by RDHP[2]. This paper describes the *in vivo* activity, serum binding and degree of RDHP-mediated hydrolysis seen with some of the 6-(substituted methylene)penems reported in our previous papers[3~5], and with a number of additional penems not previously described.

Chemistry

The syntheses of the penem salts (**3a**~**3l**) were described in Parts I~III[3~5]. The remaining penems (**3m**~**3t**) were prepared using the previously described sequence; *viz* conversion of the acetate[6,7] (**1**) to the ester (**2**) and thence to the salt (**3**)[5].

Biology

All penems tested were racemic at position C-5. Addition of a partially purified preparation of *Bacillus cereus* II β-lactamase (BC2) to any of the racemates resulted in a 50% reduction in peak height on HPLC, with no further reduction observed after prolonged incubation. A route to chiral 5-*R* derivatives was found[8] and used to prepare the chiral 5-*R* derivatives **3b** and **3k**. Treatment of these 5-*R* derivatives with BC2 resulted in complete destruction of compound, from which we concluded that BC2 was selectively hydrolysing the 5-*R* enantiomer without affecting the 5-*S* enantiomer. Moreover, the degree of synergy with amoxycillin shown by the 5-*R* enantiomer was about twice that seen with the racemate, whereas the 5-*S* enantiomer (*i.e.* the BC2-treated racemate) was a weak synergist of amoxycillin (Table 1). Thus, the



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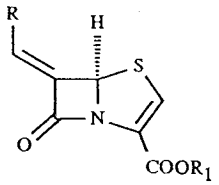
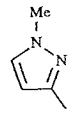
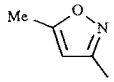


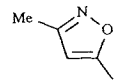
2 R_1 = PNB
3 R_1 = Na

3a R = Me

3b R = 

3c R = 

3d R = 

3e R = 

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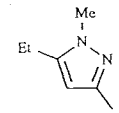
3g R = 

3h R = 

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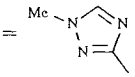
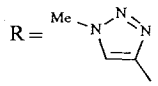
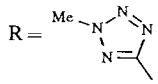
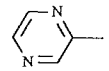
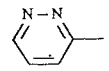
3j R = 

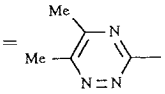
3k R = 

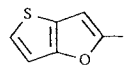
3l R = 

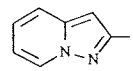
3m R = Ph

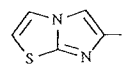
3n R = 

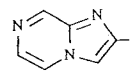
3o R = 

3p R = 

3q R = 

3r R = 

3s R = 

3t R = 

All compounds are racemic, only one enantiomer has been depicted.

Table 1. Antibacterial activity of amoxycillin alone and in the presence of 1 μg/ml of racemate **3b** and the individual 5-*R* and 5-*S* enantiomers.

Inhibitor[a]	Amoxycillin MIC (μg/ml)	
	Enterobacter cloacae Ia[b]	*Escherichia coli* (TEM-1) III
None	512	>512
3b (5-*R*,*S*)	16	2
5-*R*	4	1
5-*S*	256	32

[a] **3b** and both enantiomers had MICs of >32 μg/ml against both organisms.
[b] Classification of RICHMOND and SYKES[13].

5-*R* enantiomer would seem to be the biologically active component.

It was therefore possible to distinguish between the individual enantiomers by assaying with and without the addition of BC2 β-lactamase. In this way, the concentration of total compound and of 5-*S* enantiomer could be measured, and the concentration of 5-*R* enantiomer deduced by difference.

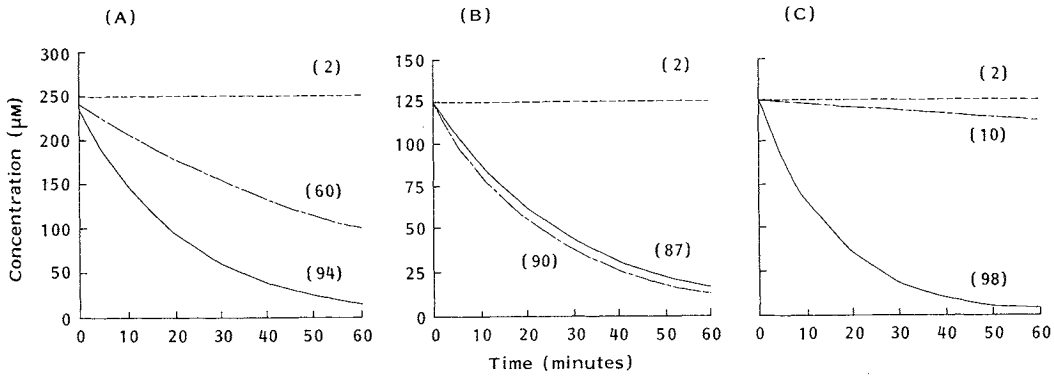
Early studies on these compounds showed that many were rapidly degraded by human kidney homogenate (HK). The furan derivative **3b** was therefore selected for detailed investigation, and a 250-μM solution of **3b** in HK was incubated at 37°C with regular sampling over a 60-minute period. A second solution of **3b** in HK also contained the RDHP inhibitor cilastatin, so that the contribution of RDHP to HK stability could be deduced. The data obtained are shown in Fig. 1A~1C.

3b was stable in MOPS buffer for 60 minutes at 37°C (Fig. 1A) but was almost totally hydrolysed in HK. Inclusion of an equimolar concentration of the RDHP inhibitor cilastatin in the reaction mixture reduced the amount of **3b** lost from 94% to 60% but further increase in the cilastatin concentration up to 10×equimolar concentration, gave no additional protection (data not shown).

Pre-treatment of **3b** with BC2 gave a solution of 5-*S* enantiomer which decayed rapidly in HK, with approx 90% lost irrespective of the cilastatin concentration present (Fig. 1B). Thus, a major part of the non-RDHP-mediated degradation of **3b** was due to loss of 5-*S* enatiomer. The 5-*R* enantiomer showed the same rapid decay in HK (98% loss in 1 hour), but in this case cilastatin gave substantial protection (Fig. 1C). Slight breakdown (10%) was still seen, however, as a result of some non-RDHP-mediated mechanism.

Fig. 1. Stability of **1b** in HK and/or MOPS buffer (% loss of **1b** in 1 hour).

(A) Racemate, (B) 5-*S* enantiomer, (C) 5-*R* enantiomer. --- MOPS buffer, ——— HK + cilastatin, —— HK.



Both enantiomers are stable in MOPS buffer, but hydrolysed in the presence of HK. Cilastatin (250 μM) minimises the loss of 5-*R* enantiomer (C) but not 5-*S* enantiomer (B).

Table 2. Antibacterial activity of amoxycillin in the presence of 1 μg/ml inhibitor.

Inhibitor[a]	Amoxycillin MIC (μg/ml)				
	E.cl. Ia[b]	*P.m.* II	*E.co.* (TEM-1) III	*K.p.* IV	*E.co.* (OXA-1) V
None	512	>512	>512	256	>512
3a	256	>256	4	2	NT
3b	16	4	2	4	32
3d	2	4	8	8	16
3j	32	4	16	1	8
3m	256	>512	64	128	NT
3n	32	4	2	8	NT
3o	8	4	4	8	16
3p	128	2	8	16	256
3q	4	8	4	8	32
3r	64	8	32	16	64
3s	8	2	2	4	64
3t	128	8	32	8	>512
Clavulanic acid	>512	16	8	4	>512
Sulbactam	256	64	128	64	>512
Tazobactam	256	16	8	16	>512

MICs of inhibitors alone were >32 μg/ml.

[a] Compounds **3c**~**3g**, **3i**, **3k**~**3l** referred to in ref 5.

[b] β-Lactamase classification based on RICHMOND and SYKES[13].

Abbreviations: *E.cl.*, *Enterobacter cloacae*; *P.m.*, *Proteus mirabilis*; *E.co.*, *Escherichia coli*; *K.p.*, *Klebsiella pneumoniae*.

NT: Not tested.

The HK stability of all subsequent penem derivatives was determined from only two samples, taken at 0 and 1 hour. BC2 enzyme was used to determine the relative stabilities of the two enantiomers, but cilastatin was not included routinely in these tests.

Most of the penems reported here were potent inhibitors of a wide range of bacterial β-lactamases[3~5] as evidenced by the improvement in the activity of amoxycillin against β-lactamase producing organisms

Table 3. Stability in HK and binding to human serum.

Compound	Kidney stability (% remaining at 1 hour)		Binding to human serum (%)	Compound	Kidney stability (% remaining at 1 hour)		Binding to human serum (%)
	5-*R*	5-*S*			5-*R*	5-*S*	
3a	10	<1	98	**3k**	70	2	68
3b	5	5	97	**3l**	16	<1	71
3c	>1	24	93	**3m**	35	3	>98
3d	50	<1	96	**3n**	29	<1	94
3e	17	30	88	**3o**	20	9	67
3f	24	3	90	**3p**	5	<1	NT
3g	<1	<1	82	**3q**	79	3	>98
3h	12	<1	94	**3r**	53	<1	93
3i	1	<1	86	**3s**	83	12	94
3j	48	<1	71	**3t**	54	<1	89

NT: Not tested.

seen in the presence of only 1 μg/ml of penem (Table 2). However, the stability of these compounds in HK varied considerably, with all compounds showing some breakdown, and a few showing complete loss within 1 hour at 37°C (Table 3). In general, the 5-*S* epimers proved less stable than the 5-*R*, but since β-lactamase inhibitory activity is found predominantly in the 5-*R* enantiomers, the reasons for poor stability of the 5-*S* enantiomers were not studied in detail.

Table 4. CD_{50} of amoxycillin in the presence of 2 mg/kg inhibitor against a TEM-1 producing *Escherichia coli* infection in mice.

Compound	CD_{50} (mg/kg × 2)
None	>100
3a	>100
3k	7
3q	>100
3s	>100
3t	>100
Clavulanic acid	40
Tazobactam acid	40
Sulbactam	>100

CD_{50} of all inhibitors alone >100 mg/kg × 2.

The ethylidene derivative (**3a**) showed substantial degradation of the active 5-*R* enantiomer in the presence of HK, as did the majority of 5-heterocyclic derivatives (**3b**~**3l**). However, **3k** proved particularly stable, with 70% of the 5-*R* enantiomer surviving a 1-hour exposure to HK. Inclusion of a 15-fold molar excess of cilastatin had no effect on the recovery of 5-*R* in this case (data not shown), suggesting that the 30% loss was not due to RDHP hydrolysis.

A number of derivatives were made in which the substituent was present as a six-membered aromatic ring (**3m**~**3p**). Some of these proved to be good synergists of amoxycillin *in vitro* (Table 2), although all compounds of this type proved susceptible to RDHP hydrolysis (Table 3).

Some of the penem derivatives bearing a bicyclic aromatic substituent were found to be very effective synergists with amoxycillin (Table 2) and to have good stability to RDHP (*e.g.* **3q** and **3s**). However, all of these bicyclic derivatives (**3q**~**3t**) were highly bound in human serum. Furthermore, all of those tested in a mouse infection model had poor *in vivo* activity (Table 4).

Thus, the N1-methyltriazolylmethylene derivative (**3k**) was selected as the compound with the best overall activity and stability. This derivative had good synergistic activity with amoxycillin *in vitro* and *in vivo*[5], was only moderately bound (68%) in human serum and was not metabolised to any great extent by RDHP. The chiral synthesis of this compound is described elsewhere[8] and the biological activity of a number of chiral N1-substituted-triazolylmethylene derivatives will be the subject of a further publication.

Table 5. Spectral data of penem esters (**2m**~**2t**).

Compound	IR ν_{max} ($CHCl_3$) cm^{-1} (β-lactam)	UV λ_{max}^{EtOH} nm (ε_m)	^1H NMR ($CDCl_3$)			
			2-CH[a]	5-CH[b]	8-CH[b]	Heterocycle
2m	1775	300 (27,210)	7.36	6.66	7.20	7.30~7.36 (2H, m), 7.43~7.50 (3H, m)
2n	1780	250 (19,800), 309 (18,600)	7.70	6.75	7.39	8.66 (1H, d, J=2.4 Hz), 8.75~8.77 (1H, m), 8.92 (1H, d, J=1.4 Hz)
2o	1785	260 (19,000), 310 (7,940)	7.41	6.74	7.12	7.63~7.51 (2H, m), 9.12~9.21 (1H, m)
2p	1785	262 (30,500)	7.39	6.66	7.34	2.60 (3H, s), 2.75 (3H, s)
2q	1775	350 (23,000)	7.06	6.67	7.40	7.00 (1H, d, J=1 Hz), 7.09 (1H, d, J=5.5 Hz), 7.44 (1H, d, J=5.5 Hz)
2r	1780	285 (30,195)	7.22	6.67	7.40	6.65 (1H, s), 6.86 (1H, t, J=7.0 Hz), 7.17 (1H, t, J=7.0 Hz), 7.57 (1H, d, J=9.0 Hz), 8.43 (1H, d, J=7.0 Hz)
2s	1775	312 (30,300)	7.30[c]	6.72	7.83	7.38 (1H, d, J=4.3 Hz), 7.99 (1H, d, J=4.3 Hz), 8.20 (1H, s)
2t	1790	—	7.55[c]	6.80	7.90	7.96 (1H, d, J=4.6 Hz), 8.48 (1H, s), 8.66 (1H, d, J=4.6 Hz), 9.10 (1H, s)

[a] s.
[b] Either br s or d, J=0.5~1.0 Hz.
[c] DMSO-d_6 solvent.

Table 6. Spectral data of penem salts (**3**).

Compound	UV $\lambda_{max}^{H_2O}$ nm (ε_m)	^1H NMR (D_2O)			
		2-H[a]	5-H[b]	8-H[b]	R
3m	290 (26,120), 365 (1,950)	7.04	6.78	7.23	7.43~7.53 (5H, m)
3n	246 (13,300), 299 (12,200)	7.04	6.67	7.24	8.51 (1H, d, J=2.4 Hz), 8.69 (1H, br s), 8.73 (1H, br s)
3o	252 (14,330)	7.03	6.64	7.25	7.80~7.85 (2H, m), 9.08 (1H, m)
3p	260 (16,900)	7.06	6.65	7.20	2.60 (3H, s), 2.65 (3H, s)
3q	359 (19,200)	6.97	6.54	7.13	6.94 (1H, s), 7.08 (1H, d, J=5.3 Hz), 7.53 (1H, d, J=5.3 Hz)
3r	282 (25,948), 352 (5,521)	6.94	6.53	7.00	6.43 (1H, s), 6.85~6.95 (1H, m), 7.17~7.23 (1H, m), 7.54 (1H, d, J=8.9 Hz), 8.37 (1H, d, J=6.3 Hz)
3s	310 (24,750)	6.97	6.54	7.03	7.10 (1H, d, J=4.5 Hz), 7.63 (1H, d, J=4.5 Hz), 7.90 (1H, s)
3t	221 (16,100), 261 (16,550), 308 (11,250)	6.98	6.62	7.17	7.82 (1H, d, J=4.7 Hz), 8.14 (1H, s), 8.35 (1H, d, J=4.7 Hz), 8.93 (1H, s)

[a] s.
[b] Either br s or d, J=0.5~1.0 Hz.

Experimental

All compounds were synthesised in our Research Laboratories, except for cilastatin, which was a gift from Merck Sharp & Dohme Research Laboratories. The preparation of the penems (**3m**~**3t**) has

been described in patent applications[9,10]. The penem esters (**2m**～**2t**) were prepared from the acetates (**1m**～**1t**)[6,7] using the general elimination procedure described in Part II[4]. The spectral data for these compounds are shown in Table 5. The sodium salts (**3m**～**3t**) were obtained from the corresponding esters (**2**) using the general deprotection method described in Part II[4]. The spectral data for these compounds (Table 6) showed that the salts were homogeneous and contained up to 15% water.

MICs were determined in microtiter plates by serial dilution of amoxycillin in broth, followed by addition of inhibitor (1 μg/ml) and organism (approx 2×10^6 cfu/ml). MIC values were recorded after incubation at 37°C for 18 hours[11].

HK was prepared as described previously[12], and compounds were assayed by reverse phase HPLC on a Waters MicrobondaPak C18 RP column. Compounds were eluted with acetonitrile in 0.1 M ammonium dihydrogen orthophosphate buffer (pH 4.7), with detection at λ_{max} wavelength. Compounds were used at a concentration of 250 μM and incubated at 37°C in 0.02 M MOPS buffer or in HK. For the initial studies, samples were assayed at 0, 5, 10, 20, 30, 45 and 60 minutes, but for the data reported in Table 3 samples were assayed at 0 and 60 minutes only.

To determine serum binding, compounds were diluted in pooled human serum to a final concentration of 100 μg/ml and solutions were then left at room temperature for 15 minutes, following which the bound fraction was separated from the free fraction using an Amicon Micropartition System centrifuged at 2,500 rpm for 20 minutes. Compounds were then assayed by HPLC, as described above.

50%-Curative dose data (CD_{50}) were carried in mice, as previously described[4]. This test had a high level of reproducibility with amoxycillin giving a typical CD_{50} of 7.2 ± 1.9 mg/kg in the presence of 2 mg/kg **1k**.

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References

1) KAHAN, F. M.; H. KROPP, J. G. SUNDELOF & J. BIRNBAUM: Thienamycin: Development of imipenem-cilastain. J. Antimicrob. Chemother. 12 (Suppl. D): 1～35, 1983
2) MATSUDA, K.; K. SASAKI, K. INOUE, H. KONDO, M. INOUE & S. MITSUHASHI: In vitro antibacterial activity of Sch 34343 and its stability to β-lactamases and renal dehydropeptidase 1. Antimicrob. Agents Chemother. 28: 684～688, 1985
3) BASKER, M. J. & N. F. OSBORNE: 6-(Substituted methylene)penems, potent broad spectrum inhibitors of bacterial β-lactamase. I. Racemic 6-ethylidenepenems. J. Antibiotics 43: 70～75, 1989
4) BROOM, N. J. P.; K. COLEMAN, P. A. HUNTER & N. F. OSBORNE: 6-(Substituted methylene)penems, potent broad spectrum inhibitors of bacterial β-lactamase. II: Racemic furyl and thienyl derivatives. J. Antibiotics 43: 76～82, 1989
5) BENNETT, I. S.: N. J. P. BROOM, G. BRUTON, S. CALVERT, B. P. CLARKE, K. COLEMAN, R. EDMONDSON, P. EDWARDS, D. JONES, N. F. OSBORNE & G. WALKER: 6-(Substituted methylene)penems, potent broad spectrum inhibitors of bacterial β-lactamase. III. Structure-activity relationships of the 5-membered heterocyclic derivatives. J. Antibiotics 44: 331～337, 1991
6) OSBORNE, N. F. (Beecham): β-Lactam compounds, their preparation and use. Eur. Pat. Appl. 0 041 768, Dec. 16, 1981
7) OSBORNE, N. F. (Beecham): Novel compounds. Eur. Pat. Appl. 0 154 132, Sept. 11, 1985
8) OSBORNE, N. F.; N. J. P. BROOM, S. COULTON, J. B. HARBRIDGE, M. A. HARRIS, I. STIRLING-FRANCOIS & G. WALKER: A novel and stereocontrolled synthesis of (5*R*) (*Z*)-6-(1-methyl-1,2,3-triazol-4-ylmethylene)penem-3-carboxylic acid, a potent broad spectrum β-lactamase inhibitor. J. Chem. Soc. Chem. Commun. 1989: 371～373, 1989
9) OSBORNE, N. F. (Beecham): Novel compounds. Eur. Pat. Appl. 0 150 781, Aug. 7, 1985
10) BROOM, N. J. P.; S. COULTON, P. D. EDWARDS & N. F. OSBORNE (Beecham): Bicyclic penems. PCT Int. Appl. 005 25, Jan. 29, 1987
11) BROOKS, G.; K. COLEMAN, J. S. DAVIES & P. A. HUNTER: Synthesis and β-lactamase inhibitory activity of 9-(2-amidoethenylthio)-9-deoxy derivatives of clavulanic acid. J. Antibiotics 41: 892～898, 1988
12) BASKER, M. J.; R. J. BOON, S. J. BOX, E. A. PRESTIGE, G. M. SMITH & S. R. SPEAR: Properties of MM 13902 and other carbapenem antibiotics *in vitro* and *in vivo*. J. Antibiotics 36: 416～422, 1983
13) RICHMOND, M. H. & R. B. SYKES: The β-lactamases of Gram negative bacteria and their possible physiological role. Adv. Microb. Physiol. 9: 31～88, 1973