Structure-activity Relationship of New 2-Substituted Penem Antibiotics

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The antibacterial activities of three new penems with 4-hydroxyprolinamide, 1-prolinamide and *N*-methyl-*N*-2-propionamide substituents, respectively, in position 2 and of their stereoisomers were examined against *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli* and *Pseudomonas aeruginosa*. All substituents conferred a broad antibacterial spectrum on the penem moiety. Changes in stereoisomerism selectively improved the activity against *E. coli*, *S. aureus* or enterococci. The structure-activity relationships of each compound were discussed in relation to minimum inhibitory concentrations, penicillin-binding protein (PBP) affinity and outer membrane permeability coefficient in *E. coli*. In this microorganism, PBP 2 was the target for all compounds. Changes in stereoisomerism influenced the affinity for PBPs 1A/B and 2. All antibiotics easily permeated the outer membrane of *E. coli* and, within each group of compounds, the penetration rate correlated with the antibacterial activity.

Penems are potent synthetic beta-lactam antibiotics with a wide spectrum of antibacterial activity [1~7]. These compounds bind poorly to beta-lactamases and are not hydrolyzed by them. The small, compact size of penems contributes to their ability to cross the outer membrane of Gram-negative bacteria rapidly, and affinity for PBP 2 contributes to their activity.

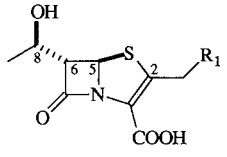
This paper deals with the biological characterization of three new penems which carry 4-hydroxyprolinamide, 1-prolinamide and *N*-methyl-*N*-2-propionamide substituent, respectively, in position 2. As a new approach to the study of structure-activity relationships we analyzed the effect of changes in the stereoisomerism of these compounds on antibacterial activity, affinity for PBPs, penetration through the outer membrane and stability to a metallo-beta-lactamase.

Materials and Methods

Compounds

A number of new 2-substituted penem derivatives, bearing a nitrogen atom linked to the bicyclic skeleton through a methylene spacer, were synthetized. The nitrogen substituents chosen from among amino acid amide derivatives (Table 1) were 4-hydroxyprolinamide (for type 1 compounds), 1-prolinamide (type 2 compounds) and *N*-methyl-*N*-2-propionamide (type 3). All different amino acid stereoisomers were synthesized. Synthesis (Scheme 1) of 2-substituted penem acids started from allyl (5*R*,6*S*)-2-hydroxymethyl-6-[(1*R*)-1-tert-butyldimethylsilyloxyethyl]-penem-3-carboxylate (**4**).[8] The primary hydroxy group in **4** was activated as the mesylate **5** and allowed to react with amino acid amides to give fully protected penems **6**. Removal of the silyl ether in position 8 and the allyl ester in position 3 finally gave

Table 1. Structures of 2-substituted penem derivatives used in this study.



Compound	R_1
1A	(2*S*,4*R*)-4-Hydroxy-1-prolinamido
1B	(2*R*,4*R*)-4-Hydroxy-1-prolinamido
1C	(2*S*,4*S*)-4-Hydroxy-1-prolinamido
1D	(2*R*,4*S*)-4-Hydroxy-1-prolinamido
2A	(2*S*)-1-Prolinamido
2B	(2*R*)-1-Prolinamido
3A	*N*-Methyl-*N*-(2*S*)-(2-propionamido)amino
3B	*N*-Methyl-*N*-(2*R*)-(2-propionamido)amino

Dedicated to the memory of Professor SATTA, who passed away on October 9, 1994.

penem acids (1A ~ D, 2A ~ B, 3A ~ B).

Bacterial Strains

The source and the main properties of the strains used in this study are reported in Table 2. Strains not indicated in Table 2 were from our own collections. Growth media were either Mueller-Hinton (MH; Difco, Detroit, Mich.) or Luria-Bertani (LB) which consisted of tryptone (Difco) 1% yeast extract (Difco) 0.5%, NaCl 1%, pH 7.5. Media were made solid by adding agar 2%. Culture media were supplemented with the appropriate antibiotic for selection and maintenance of the acquired genotypes.

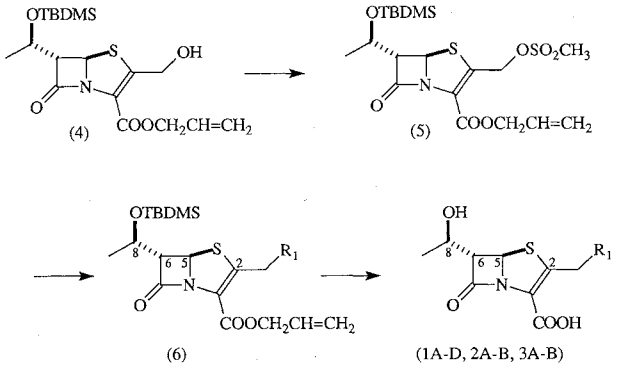
Determination of MIC

MICs were determined by the two-fold agar dilution method using MH-agar. Plates were inoculated with a multipoint device (10^4 CFU/spot) and incubated at 35°C for 24 hours. MIC was defined as the lowest antibiotic concentration which did not give any visible growth.

Morphology Studies

Bacteria were grown for 4 hours in MH-broth containing the desired antibiotic concentrations and fixed in 2% (v/v) formaldehyde (Carlo Erba). Samples were stained with methylene blue and photographed with a Leitz Orthoplan 2 microscope (Ernst Leitz, Wetzlar, Germany).

Scheme 1.



Penicillin Binding Protein (PBP) Analysis

E. coli membranes were prepared by the SPRATT method[15], resuspended in 10 mM sodium phosphate buffer (pH 7) at a protein concentration of 10 ~ 20 mg/ml and stored at −70°C. 100 μg of membrane proteins were incubated with the antibiotic at the desired concentration for 10 minutes at 37°C. Ten μg/ml of [benzyl-4-^{3}H]-penicillin ethylpiperidinium salt 25 Ci/mmol, (kindly supplied by Merck & Co., Inc., Rahway, N.Y.) were added and incubations were continued for another 10 minutes. PBP binding reactions were stopped by addition of 2% (v/v) Sarkosyl (IBI, New Haven, Conn.). SDS-polyacrylamide gel electrophoresis and fluorography were performed as described[15]. PBP fluorographs were quantitated with a scanning laser densitometer (Ultrascan XL; LKB, Bromma, Sweden).

Permeability Assays with Intact Cells

E. coli LGC10 harboring the plasmid pAA20R, which contained the gene coding for a carbapenem-hydrolyzing beta-lactamase (CphA) cloned from *Aeromonas hydrophila*[16] was used for these assays. The Michaelis constant (*Km*) for CphA was determined using the Eadie-Hofstee plot of initial velocity (v) at six different substrate concentrations. Twenty-fold diluted crude cellular extracs were used as the enzyme sources, and the hydrolysis of beta-lactam antibiotics was followed with a Beckman DU-7 UV spectrophotometer, with readings recorded at 10-second intervals for 5 minutes, operating at the wavelength at which the difference between the extinction values of the hydrolyzed and non-hydrolyzed molecule was maximum (this wavelength was 298 nm for imipenem and 310 nm for the penem derivatives). Alternatively, the *Km* values were obtained directly from a time course of the hydrolysis data, as previously

Table 2. Designation, relevant genotype, and origin of strains.

Strain	Relevant genotype	Phenotype	Reference
Escherichia coli			
C600	Wild type	Wild type	10
TEM-1	pMK20::Tn 3 trasformant of C600	TEM-1 producer	10
TEM-2	pMK20::Tn 1 transformant of C600	TEM-2 producer	10
SHV-1	pR1010 transformant of C600	SHV-1 producer	10
pop1010	*hfr his metA rpoB*	$OmpF^+$ $OmpC^+$	11
B1428	*Hfr his metA aroB rpoB ompC::Tn 5*	$OmpF^+$ $OmpC^-$	12
B1449	*Hfr his metA aroB rpoB ompF::Tn 5*	$OmpF^-$ $OmpC^+$	12
pop1389	*Hfr his metA rpoB ompB101 lac*	$OmpF^-$ $OmpC^-$	11
LGC10	pAA20R transformant of pop1010	$OmpF^+$ $OmpC^+$ $CphA^+$	13
LGC78	pAA20R transformant of B1478	$OmpF^+$ $OmpC^-$ $CphA^+$	13
LGC49	pAA20R transformant of B1449	$OmpC^+$ $OmpC^+$ $CphA^+$	13
LGC89	pAA20R tranformant of pop1389	$OmpC^-$ $OmpC^-$ $CphA^+$	13
Pseudomonas aeruginosa			
K799	Wild type	Wild type	14
K799/61	Mutant of K799	Hyperpermeable outer membrane	14

described[17,18], with no significant differences in the values obtained.

For permeability assays the cells were grown in up to the mid-exponential growth phase and harvested by centrifugation. They were washed twice with 10 mM sodium phosphate buffer (pH 7) supplemented with 5 mM $MgCl_2$, resuspended in the same buffer, and sonicated with a Labsonic 2000 ultrasonic disrupter (B. Braun Melsungen AG, Melsungen, Germany) by three to four 15-second sonication pulses with an intervening 30-seconds in ice to minimize beta-lactamase damage. The hydrolysis rates of 10 μM antibiotic by intact cells and sonic extracts of cells were obtained by spectrophotometric assay, as described above. To measure the extent of leakage of enzyme into the medium, the rate of beta-lactam hydrolysis was measured with supernatants obtained by centrifugation of the intact-cell suspension, and the intact cell hydrolysis rates were corrected for the contribution of extracellular enzyme. The permeability coefficient was calculated using the method of NIKAIDO *et al.*[19] with the modifications proposed by YOSHIMURA and NIKAIDO[20].

Results

Antibacterial Activities

Table 3 shows the antibacterial activities of the 2-substituted penems used in this study. For convenience, the antibiotics were grouped in three types according to the 2-substituent. Within each type, isomers were indicated by a letter (see also Table 1).

All substituents conferred a broad antibacterial spectrum, embracing both Gram-positive and Gram-negative bacteria, on the penem moiety. Changes in both substituent and stereoisomerism influenced the antibacterial activity, even to a significant extent, with a 4-fold or greater difference in the MICs for some compounds. In type 1 compounds, the best antibacterial activity was shown by 1A which bears the 4-hydroxyprolinamide moiety with a (2*S*, 4*R*) configuration; in type 2 by 2A, corresponding to a (*S*)-prolinamide configuration; in type 3, the (*S*) configuration improved the activity against *E. faecium* and *E. coli* SHV-1, whereas the (*R*) configuration improved the activity against *E. coli* TEM-2. In type 1 and 2 compounds, the change from the (*S*) to the (*R*) configuration of the amide group significantly decreased the activity against *E. faecalis* and *E. coli* strains, but did not affect the activity against the other species, with the exception of compound 1C which showed the lowest activity against the hyperpermeable *P. aeruginosa* K799/61. In type 3 compounds the change from the (*S*) to the (*R*) configuration was not consistently associated with decreased activity, since the (*R*) con-

Table 3. Antibacterial activity of 2-substituted penem derivatives used in this study.

Strain	MIC (μg/ml) of antibiotic							
	1A	1B	1C	1D	2A	2B	3A	3B
Staphylococcus aureus ATCC 29213	0.12	0.25	0.25	0.12	0.12	0.06	<0.03	0.03
S. aureus[a] MR09	8	8	8	16	2	4	2	2
Enterococcus faecalis ATCC 29212	2	32	16	32	2	16	4	2
Enterococcus faecium 23[a]	>64	>64	>64	>64	>64	>64	16	>64
Escherichia coli ATCC 25922	0.03	2	0.5	1	0.06	1	0.25	0.12
E. coli TEM-1	0.12	2	1	1	0.12	1	0.25	0.25
E. coli TEM-2	0.25	2	2	1	0.5	1	1	0.25
E. coli SHV-1	0.5	2	2	1	0.25	1	0.25	1
Pseudomonas aeruginosa K799	>64	>64	>64	>64	>64	>64	>64	>64
P. aeruginosa K799/61	0.25	0.5	8	0.5	0.5	0.25	1	2

[a] Our collection strains; MR, methicillin-resistant.

Table 4. Affinities for PBPs of 2-substituted penem derivatives used in this study.

Antibiotic	ID_{50} (μg/ml) for PBP					MIC (μg/ml)	Morphology at $^1/_2$ the MIC
	1A/B	2	3	4	5/6		
1A	0.5	0.05	>50	0.1	>50	0.06	Polymorphic round cells
1B	5	5	>50	0.1	>50	4	Filaments
1C	2	2	>50	0.1	>50	2	Filaments
1D	2	2	>50	0.1	>50	2	Filaments
2A	0.5	0.1	>50	0.1	>50	0.12	Polymorphic round cells
2B	2	1	>50	0.1	>50	1	Filaments
3A	1	0.5	>50	0.1	>50	0.25	Filaments
3B	1	0.5	>50	0.1	>50	0.25	Filaments

figuration appeared to favour activity against *E. coli* TEM-2.

All antibiotics were inactive against *P. aeruginosa* with a normal permeability barrier, but showed good activity against the hyperpermeable derivative. All of them also maintained good activity against *E. coli* producing some common beta-lactamases, even if the MICs of most compounds increased 2- to 4-fold in comparison with the strain not producing the enzymes: SHV-1 production affected the activity of 1A and 3B more markedly, and TEM-2 that of 2A and 3A.

Contribution of Cellular Components to Activity in *E. coli*

PBPs

The affinities of PBPs for all antibiotics were estimated in competition experiments using membranes isolated from *E. coli* K12. Table 4 shows the results of these experiments. All antibiotics bound PBPs 1A/1B, 2 and 4 but not PBPs 3 and 5/6. The ID_{50} of 1A and 2A for PBP 2 was significantly lower than for PBPs 1A/1B. Changes in stereoisomerism decreased the affinity for both PBPs 1A/B and 2, though more markedly in the case of PBP 2, so that compounds such as 1B, 1C, 1D, and 2B showed a similar affinity for all these PBPs. Changes in the 2-substituent or in stereoisomerism did not modify the affinity for PBP 4, which was the same for all compounds.

The MICs of all the antibiotics correlated well with the ID_{50} of PBP 2, confirming that this PBP was the target for these antibiotics. However, only compounds 1A and 2A caused the polymorphic round cell formation typical of the selective inhibition of PBP 2, whereas all the others formed filaments (Table 4 and Fig. 1).

Permeability Barrier

Penetration kinetics of penems into *E. coli* was studied using strain K12 transformed with the pAA20R plasmid harboring the gene encoding the CphA carbapenemase.

Measurement of kinetics parameters for all the compounds indicated that the range of *Km* and V_{max} values of the carbapenemase for the penems was fairly narrow (Table 5). Changes in stereoisomerism influenced the *Km* of groups 1 and 2 and the V_{max} of all groups. The highest hydrolysis rate was shown by compound 1B, whereas compound 3B was the most stable.

Fig. 1. Effect of compound 1A (a) and 1B (b) at $^1/_2$ the MIC on *E. coli* morphology.

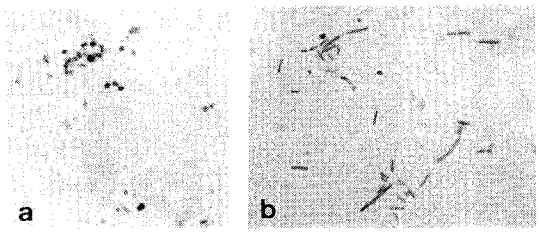


Table 5. Stability to CphA beta-lactamase and penetration rate of the penem antibiotics into *E. coli*.

Antibiotic	*Km* (μM)	V_{max} (μmol/mg/minute)	Permeability coefficient[a] (10^{-5} cm/second) ± SD
1A	372	0.390	1009 ± 210
1B	597	0.258	531 ± 130
1C	229	0.609	537 ± 118
1D	ND	ND	502 ± 94
2A	287	0.288	1196 ± 203
2B	660	0.423	546 ± 102
3A	383	0.392	966 ± 284
3B	346	0.903	1531 ± 428

[a] The permeability coefficients are averages of at least three different determination. SD, standard deviation.

Table 6. Activity of 2-substituted penem derivatives on *E. coli* strains with altered porin patterns producing or not producing carbapenemase.

Antibiotic	MIC (μg/ml)							
	Strain[a]							
	pop1010 (F^+C^+)	LGC10 (F^+C^+, $Cpha^+$)	pop1389 (F^-C^-)	LGC89 (F^-C^-, $CphA^+$)	B1449 (F^-C^+)	LGC49 (F^-C^+, $CphA^+$)	B1478 (F^+C^-)	LGC78 (F^+C^-, $CphA^+$)
1A	0.03	1	0.25	16	0.12	4	0.03	2
1B	2	2	2	64	2	16	2	8
1C	1	8	1	>64	1	64	1	16
1D	1	1	1	64	1	16	1	8
2A	0.12	1	0.5	16	0.12	2	0.12	4
2B	1	2	1	16	1	8	1	4
3A	0.25	2	0.25	64	0.25	16	0.25	32
3B	0.12	1	0.12	32	0.25	8	0.12	4

[a] The strain phenotype is in brackets; F, OmpF; C, OmpC.

The penems penetrated the outer membrane of *E. coli* at rates ranging from 502 to 1531 10^{-5} cm/second (Table 5). Among type 1 compounds, isomer 1A was endowed with a penetration rate which was double that of the other isomers. A similar difference was found between compounds 2A and 2B, whereas compound 3B penetrated 50% faster than its isomer.

The role of the permeability barrier in penem activity was also studied by determining susceptibility of *E. coli* strains with altered porin patterns and producing or not producing the CphA carbapenemase (Table 6). Lack of both porins particularly influenced the activity of compound 1A, and to a lesser extent that of 2A, but not that of any of the other compounds.

Carbapenemase synthesis was associated with an increase in MICs of all compounds for strains with both normal and altered porins, though only the latter strains expressed high levels of resistance.

Discussion

The results of this study showed that both the type of substituent and the stereoisomerism may selectively influence the activity of penems on the strains tested. The activity against *S. aureus* (both the methicillin susceptible and -resistant strain) was more influenced by the type of substituent than by the stereoisomerism whereas the latter influenced the activity of group 1 and 2 compounds against *E. faecalis* and *E. coli* as well as the activity of group 3 compounds against *E. faecium*. The *P. aeruginosa* strain with a normal permeability barrier was clearly resistant to all compounds. However, the susceptibility of the hyperpermeable mutant suggested that resistance of *P. aeruginosa* to penems is due more to poor penetration than to the low affinity of these antibiotics for their targets. All compounds were apparently endowed with good stability to TEM- and SHV-type beta-lactamases since all were active against strains producing these enzymes.

In *E. coli* all penems and carbapenems thus far described bind PBP 2 with the highest affinity but also interact with relatively good affinity with PBP 1A/B and, in the case of meropenem, with PBP 3[21], as well. The morphological change of rod shaped bacteria into ovoid cells induced by these antibiotics is ascribed to the selective inhibition of PBP 2.

The results described in Table 4 show that both the substituent and stereoisomerism may influence penem affinity for PBP 2 and 1A/B, as well as strain morphology. Ovoid cell formation was the morphological effect of compounds (1A, 2A) which had an ID_{50} for PBP 2, 5 to 10 times lower than for PBP 1A/B. At the MIC, these compounds inactivated PBP 2 only. Other compounds had a similar affinity for both PBPs and, in addition to PBP 2, inactivated a substantial amount of PBP 1A/B at the MIC, causing a completely different morphological effect, *i.e.* filaments formation. This supports the hypothesis that PBP 1A/B plays a particular role in septum formation, and our findings demonstrate that this role cannot be taken over by PBP 3.

Changes in both the substituent and stereoisomerism caused a variation in stability to the specific beta-lactamase, CphA carbapenemase (Table 5). The majority of the tested compounds showed a *Km* ranging from 229 to 383 μM; these values were fairly similar to those measured for imipenem (281 μM)[13], except that higher values were found for the compounds 1B (597 μM) and 2B (660 μM), which, in turn, were closed to the *Km* values reported for meropenem (962 μM)[13]. The range of V_{max} values was also similar to those previously measured for both imipenem and meropenem[13].

Penetration rate through the outer membrane is another important factor in determining the activity of beta-lactams. All the penems penetrated the outer membrane of *E. coli* at rates which were broadly one order of magnitude lower than those measured in the same strain for carbapenems[13]. However, these rates were sufficiently high to allow compounds to be relatively active against strains producing CphA carbapenemase (Table 6). Table 6 also shows that defects in porins affected the activity of 1A and 2A more significantly than that of the other compounds. This suggested that porins cannot be the only factor governing penetration of penems, but that other mechanisms may exist. A similar hypothesis has already been adduced to explain the different effects of porin defects on susceptibility of *E. coli* to imipenem and meropenem[13]. As expected, the simultaneous presence of an active beta-lactamase and alteration of porins caused a substantial decrease in susceptibility of strains to all compounds (Table 6).

Since a permeability barrier does not exist in Gram-positive bacteria, the activity of penems against *S. aureus* and enterococci essentially reflect their affinity for essential PBPs including those PBPs such as PBP 2a of methicillin-resistant staphylococci and PBP 5 of enterococci which are involved in the resistance of these microorganisms to beta-lactams[22~27]. The activity of penem derivatives against *S. aureus* MR 09, *E. faecalis* and *E. faecium* indirectly suggest (i) that both isomers of substituent 3 may have good affinity for PBP 2a of staphylococci; (ii) that substituents 1A, 2A and 3B may have good affinity for PBP 5 of *E. faecalis* and (iii) that substituent 3A may have good affinity for PBP 5 of *E. faecium*.

In conclusion, the data presented here stress the importance of this approach to study the structure-activity relationship of new beta-lactams by showing that minute changes in the steric configuration of the ligand can profoundly modify its interaction with the cellular components involved in beta-lactam activity. An understanding of the effects of these minor changes may provide useful information for the design of new types of inactivators of penicillin-interactive proteins and

domains.

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