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PENEM INHIBITORS OF BACTERIAL SIGNAL PEPTIDASE

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Abstract: C(3)-Penem esters and amides having the (5*S*)-configuration at the bridgehead are inhibitors of *Escherichia coli* leader peptidase, the best activity being seen with a 6-(1-acetoxyethyl) derivative having the (5*S*, 6*S*, 8*R*)-stereochemistry. These compounds represent the first examples of potent inhibitors of bacterial signal peptidase.

Introduction

Protein secretion in bacteria is rapidly becoming a pathway of widespread interest for the derivation of antibacterial agents[1]. Signal peptidases were the first such targets to be acknowledged, and over the past four years have received attention from a variety of authors [2,3,4]. Their physiological function is to ensure release of secreted proteins from the outer surface of the plasma membrane by removal of the signal sequence or cleavable membrane anchor from the preproteins[5]. In *E.coli* there appears to be an essential enzyme, leader peptidase (LP), which fulfils this function for most proteins destined either for the periplasmic space or the outer membrane. [6,7] Leader peptidase is anchored to the membrane by two hydrophobic domains close to the *N*-terminus, and the catalytic portion of the molecule is located in the periplasm.[8]

Leader peptidase is a highly specific enzyme which is capable of recognizing a distinct site within substrate preproteins. However sequences around cleavage sites are not highly conserved and consequently the structural elements which govern recognition by leader peptidase are still unclear.[9] Natural substrates are not attacked at realistic rates *in vitro*, without manipulation either to prevent or destabilise folded conformations. Leader peptidase can however recognise, and specifically cleave, peptides based on full length preproteins which also contain consensus cleavage sites, without any additional treatment. Assays of this type based on the work of Dev and Ray[2] have been used to measure the potency of inhibitors in this study.

Enzyme Assay

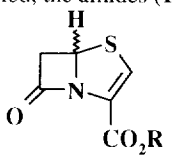
Leader peptidase was prepared according to the method of Zwizinski and Wickner[10] from a strain containing the plasmid pRD8.[11] Two synthetic peptides have been used for assays: Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-NH_2 (A); the other differing only in replacement of the *N*-terminal Phe by Trp (B). In both cases cleavage of peptides was monitored using a hplc gradient system with UV detection at 215 nm. Conditions were similar to the published method[2] with the following modifications: gradients were created between H_2O/0.1%TFA(a) and CH_3CN/0.08%TFA(b); stabilisation at 15% b, initialisation 15-20% b, gradient 20-46% b over 13 min and column washing 46-90% b, at a flow rate of 0.2 ml/min. Assays using substrate (A) (**Tables 1** and **2**) were carried out at 500 μM peptide and 2 μM enzyme concentrations, with 15 min pretreatment of enzyme with inhibitor or 5% DMSO (as control). Reactions were incubated at 37°C and stopped with 0.15% TFA when

30% cleavage was achieved. Substrate (B) was used for I_{50} estimations as indicated (**Table 2**) at a peptide concentration of 500 μm, and 25 nM enzyme concentration under similar conditions (approximate K_m for this substrate 0.3 mM compared to 1 mM for substrate A).

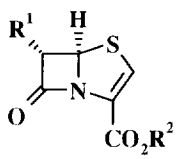
Chemistry and SAR of Inhibitors

When work on inhibitors of LP commenced in these laboratories, very little was known about the catalytic centre of the protease and a variety of known protease inhibitor classes were tested in the assay without any significant inhibition of the enzyme being observed. The strategy was changed when it was found that LP was a serine protease rather than one of the other three classes[3,12] and moreover was based on serine/lysine rather than the classical serine/histidine/aspartate triad.[13] A large collection of beta-lactam containing compounds was available that were developed as inhibitors of bacterial beta-lactamases, transpeptidases and carboxypeptidases; an enzyme family that is also based on a serine/lysine catalytic dyad. Therefore a range of these compounds carrying free acid and ester substituents was screened against leader peptidase with the result that the racemic penem benzyl ester (**1**)[14] was found to inhibit the enzyme.

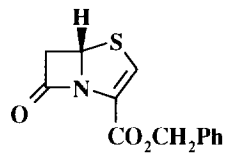
To investigate the scope of this initial finding, a program of synthesis of penems was initiated.[15] The penem 4-nitrobenzyl ester (**2**) was prepared from 4-acetoxyazetidin-2-one using the phosphorane cyclisation methodology[14] and formed the basis for a divergent synthetic strategy for a range of other esters. Catalytic hydrogenation of the ester (**2**) produced the free acid (**3**), which was esterified with the appropriate alkyl halide and potassium carbonate in *N,N*-dimethylformamide (DMF) to produce the penem esters (**8 - 17**). In order to determine the effect of penem chirality on the observed inhibition of LP, the separate enantiomers were required. The (5*R*)-6-bromopenem (**4**) was prepared from 6-aminopenicillanic acid[16] and reduced with zinc and ammonium acetate to give the (5*R*)-penem 4-methoxybenzyl ester (**5**). Ester exchange of penem (**5**) was carried out using aluminium chloride/anisole deprotection followed by reaction of the sodium salt with benzyl bromide in DMF to give the benzyl ester (**6**), $[\alpha]_D^{23}$ +201°(c 1.0 CH_2Cl_2). The Woodward route[17] involving fractional crystallisation of an intermediate (-)-menthyl ester was employed to obtain the (5*S*)-penem benzyl ester (**7**), $[\alpha]_D^{23}$ -206°(c 1.0 CH_2Cl_2). The possibility of ester hydrolysis by serum esterases was considered a potential problem and therefore penem amides were also examined as inhibitors. Little has been published on this class of derivatives, and the best route that we devised involved formation of a mixed anhydride from the acid (**3**) and diphenylphosphoryl chloride at -15°C followed by reaction with the required amine. By this method, the amides (**18 - 25**) were obtained.



(**1**) R = CH_2Ph
(**2**) R = $CH_2C_6H_4$-4-NO_2
(**3**) R = H



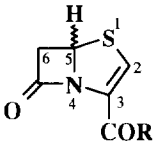
(**4**) R^1 = Br, R^2 = $CH_2C_6H_4$-4-OMe
(**5**) R^1 = H, R^2 = $CH_2C_6H_4$-4-OMe
(**6**) R^1 = H, R^2 = CH_2Ph



(**7**)

Inhibitory activities for the derivatives of pen-2-em-3-carboxylate against *E. coli* leader peptidase are shown in **Table 1**. It is immediately obvious that the stereochemistry at the bridgehead is critical. Compounds (**5**) and

Table 1

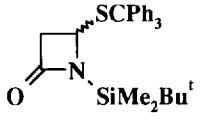


Compound	R	C5	% Inhibition at 100 μM	I_{50} μM
1	OCH_2Ph	*RS*	100	10
6	OCH_2Ph	*R*	0	ND
7	OCH_2Ph	*S*	100	3
2	$OCH_2C_6H_4$-4-NO_2	*RS*	85	50
8	$OCH_2C_6H_4$-4-OMe	*RS*	100	7
5	$OCH_2C_6H_4$-4-OMe	*R*	0	ND
9	$OCH_2C_6H_4$-4-CO_2Me	*RS*	100	3
10	$OCH_2C_6H_4$-3-CO_2Me	*RS*	72	8
11	$OCH_2C_6H_4$-4-CO_2Na *	*RS*	31	ND
12	OMe	*RS*	100	4
13	$OC_5H_{11}{}^n$	*RS*	100	21
14	$OC_{16}H_{33}{}^n$	*RS*	0	ND
15	$OCH_2CH_2CH_2CH_2Ph$	*RS*	83	13
16	OCH_2OMe	*RS*	100	6
17	$OCH_2CH{=}CH_2$	*RS*	93	10
3	OH †	*RS*	0	600
18	$NHCH_2Ph$	*RS*	34	ND
19	$NMeCH_2Ph$	*RS*	88	50
20	NHC_6H_4-4-OMe	*RS*	77	87
21	NHPh	*RS*	86	58
22	NEt_2	*RS*	86	58
23	$NHBu^t$	*RS*	0	ND
24		*RS*	93	10
25		*RS*	79	115

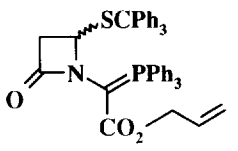
* prepared by hydrogenation of corresponding 4-nitrobenzyl ester

† as K salt; ND-not determined

(**6**), which have the (5*R*)-configuration that is present in most naturally occurring ß-lactams, and is required for inhibition of transpeptidases, do not have any activity against LP. All of the activity originally observed for the racemic benzyl ester (**1**) was due to the (5*S*)-isomer (**7**). Overall, the esters are better than the amides, with the free acid (**3**) being only very weakly active. In the ester series, a range of structural types is tolerated, but two features tend to result in decreased activity. Firstly, in the alkyl esters there is a loss of potency with



(26)



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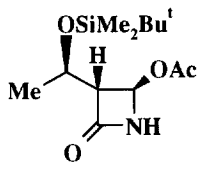

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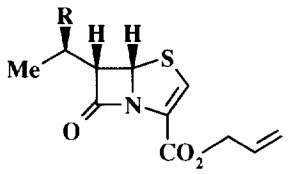
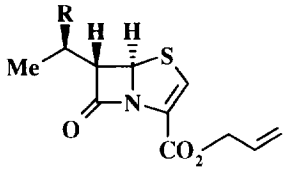
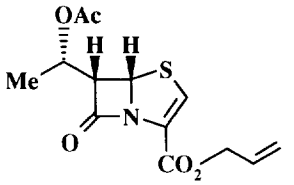


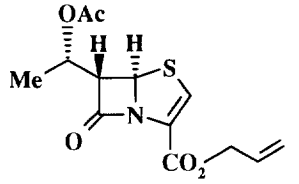
(29) R = H, racemate
(30) R = OAc, racemate
(31) R = OAc
(32) R = $OSiMe_2Bu^t$
(33) R = OH



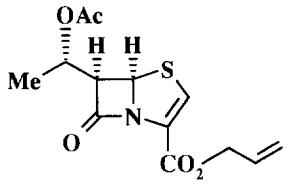
(34) R = H, racemate
(35) R = OAc, racemate
(36) R = $OSiMe_2Bu^t$



(37) racemate



(38) racemate



(39)

increasing chain length as can be seen by comparing the methyl (**12**), pentyl (**13**) and hexadecyl esters (**14**). Secondly, an acid substituent on the benzyl ester in compound (**11**) is detrimental to activity. The only amide that produced a comparable level of inhibition to the better esters was the pyrrolidine (**24**). The penem ring system tends to be hydrolytically unstable, but there are certain known modifications such as alkyl substitution at C6 that can improve this feature. The (5*RS*, 6*SR*)-6-ethylpenem (**34**) was obtained from the reaction of the azetidin-2-one (**26**)[18] with lithium di-isopropylamide (LDA) and ethyl iodide followed by construction of the 5-membered ring using standard phosphorane procedures. Heating penem (**34**) in toluene at 100°C for 4.5 h led to racemisation at the bridgehead and formation of a 1 : 1 mixture of *cis* and *trans* penems from which the (5*RS*, 6*RS*)-6-ethylpenem (**29**) was isolated by preparative h.p.l.c.. The 6-(hydroxyethyl) substituent is widely used in the field of penem inhibitors of transpeptidases and therefore the utility of this type of side-chain in the

area of LP inhibitors has been determined. Reaction of the azetidinone (**26**) with LDA and acetaldehyde followed by acetylation and construction of the thiazoline ring produced the (5*RS*, 6*SR*, 8*RS*) (**35**) and (5*RS*, 6*SR*, 8*SR*) (**38**) diastereoisomers of the 6-(acetoxyethyl)penem. Similar aldol strategy on the phosphorane (**27**)[19] gave the (5*RS*, 6*RS*, 8*SR*) (**30**) and (5*RS*, 6*RS*, 8*RS*) (**37**) isomers in addition to the *trans*-compound (**38**).

Table 2 shows the activities of the 6-substituted penems against LP. The effect of the *trans*-6-ethyl substituent in compound (**34**) has been to remove activity, but the *cis*-6-ethylpenem (**29**) is only marginally inferior to the 6-unsubstituted compound (**17**). The activity associated with the set of diastereoisomeric 6-(acetoxyethyl)penems is essentially confined to the (5*RS*, 6*RS*, 8*SR*)-compound (**30**) and this exhibits improved activity over the corresponding unsubstituted compound (**17**). This result provided encouragement to obtain the separate enantiomers of the active racemate, and in the first instance this was achieved by preparative chiral h.p.l.c. of the penem (**30**) on a Chiralpak AD column using ethanol/hexane (70 : 30) as eluant. As can be seen from **Table 2**, the activity resided with the (5*S*, 6*S*, 8*R*)-isomer (**31**), $[\alpha]_D^{24}$ -204°(c 0.9 $CHCl_3$); the (5*R*, 6*R*, 8*S*)-isomer (**39**), $[\alpha]_D^{24}$ +196°(c 1.0 $CHCl_3$), being essentially inactive. A better route to the required (5*S*)-isomer (**31**) was from the commercially available homochiral azetidinone (**28**). Standard methodology provided the (5*R*)-penem (**36**) which was subjected to irradiation[20] through Pyrex in a Hanovia medium pressure UV photoreactor to give a ~1:1 ratio of penems (**32**) and (**36**) from which the required (5*S*)-compound (**32**) was isolated and the (5*R*)-isomer (**36**) recycled. Removal of the silyl protecting group from penem (**32**) gave the alcohol (**33**), which was also a good inhibitor of LP. This material could be acetylated to produce the penem (**31**) or otherwise derivatised.

Table 2

Compound		% Inhibition at 100 μM	I_{50} μM
29	6-ethyl racemates	84	20
34		19	ND
30	6-(acetoxyethyl) racemates	100	1.8
35		64	ND
37		0	ND
38		0	ND
31	6-(acetoxyethyl) enantiomers	100	0.85 (0.07)
39		20	ND
33	6-(hydroxyethyl) 5*S* enantiomer	100	0.92 (0.18)

(Figures in brackets represent I_{50} values determined using substrate B at 25 nM enzyme concentration)

A recent paper[21] from a Merck group has reported monocyclic azetidinones as inhibitors of LP, but inhibition was only obtained at the 500 μM level. The penems (**31**) and (**33**) are the first examples of potent inhibitors of leader peptidase and are effective at concentrations close to the concentration of the enzyme in the *in vitro* assay.

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