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THE SYNTHESIS AND SERINE ß-LACTAMASE INHIBITORY ACTIVITY OF SOME PHOSPHONAMIDATE ANALOGUES OF DIPEPTIDES

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ABSTRACT: The dipeptidyl phosphonamidate analogues (**4**)-(**12**) inhibited a range of serine ß-lactamases, being most efficacious against the *Enterobacter cloacae* P99 isolated enzyme. Synergy experiments demonstrated that the antibacterial activity of amoxycillin is potentiated against bacteria producing these enzymes, the effect again being most pronounced against the P99-producing *E.cloacae* N-1 strain. The analogues (**11**) and (**12**) (ß-amino acid C-terminal) were the most active inhibitors and synergists.

Introduction

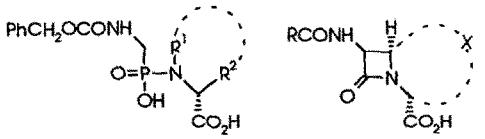
Clavulanic acid[1] is a serine ß-lactamase inhibitor of great commercial importance. Its mechanism of acylative interaction with these enzymes, resulting in their inhibition, is well understood.[2-3] Other inhibitor classes studied in these laboratories include the highly potent 6-alkylidene penem series[4] and a rational mechanism for their interaction with serine ß-lactamases has recently been proposed.[5]

Linear, substrate-related peptidyl α-amino phosphonic acid derivatives have been demonstrated[6-8] to inhibit serine proteases by the transition state isostere mechanism, since they mimic the tetrahedral intermediates formed during normal substrate hydrolysis. Other tetrahedral phosphorus compounds operate by phosphorylation/phosphonylation at the active site serine.[9-12] The recent disclosures by Pratt[13] and by Laws, *et.al*[14] of some simple phosphonamidates as inhibitors of serine ß-lactamases now prompt us to report our own findings in this area.

Synthesis of Inhibitors

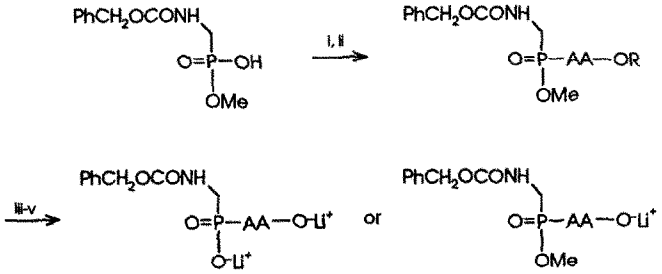
Linear phosphonamidates (**1**) resemble closely the generalised substrates (**2**) of ß-lactamases, which contain an azetidinone ring (**Figure 1**). We chose to examine a series of dipeptidyl phosphonamidate analogues as ß-lactamase inhibitors; these were obtained (**Scheme 1**) by methods described by Jacobsen and Bartlett.[15] ['AA' represents both primary and secondary amino acid residues (including prolines and the penicillin-related thiazolidine)]. These were chosen as the C-terminal of the series (**1**) to incorporate increasingly similar recognition elements R^1,R^2 associated with substrates (**2**). In the case of the cyclic variants (**8**)-(**10**) (secondary AA C-terminal) we were unable to achieve further hydrolysis of the methyl phosphonate ester function without disruption of the phosphonamidate linkage. Ethyl phosphonamidates corresponding to (**9**) (L-stereochemistry) have been described by Laws, *et.al.*[14] The L-Phe compound (**7**) is an inhibitor of carboxypeptidase A.[15]

We also prepared some 'expanded' phosphinate-type transition state analogues[17] in the hope of mimicking a late ß-lactam hydrolysis transition state. The synthesis of a representative compound (**17**) from aminophosphinic acid (**13**) is outlined in **Scheme 2**.

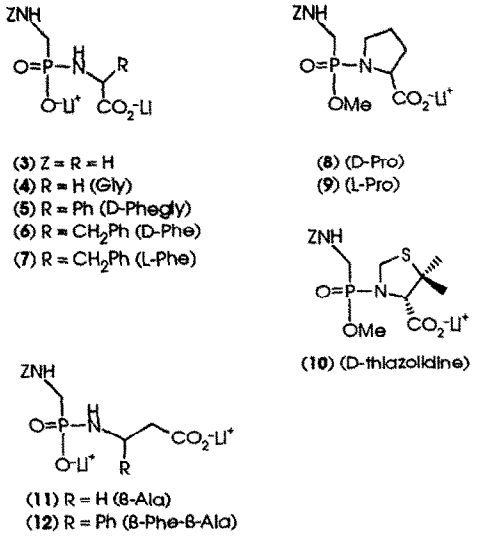


(1) (2)

Figure 1: *Relationship of linear phosphonamidates (1) to generalised β-lactam substrates (2) of serine β-lactamases.*



Scheme 1: *Synthesis of linear phosphonamidates (3)-(12):* ***Reagents:*** *i. $SOCl_2$, CH_2Cl_2; ii. AA ester[16]; iii. LiOH, H_2O-CH_3CN; iv. HP20SS chromatography; v. lyophilisation.*



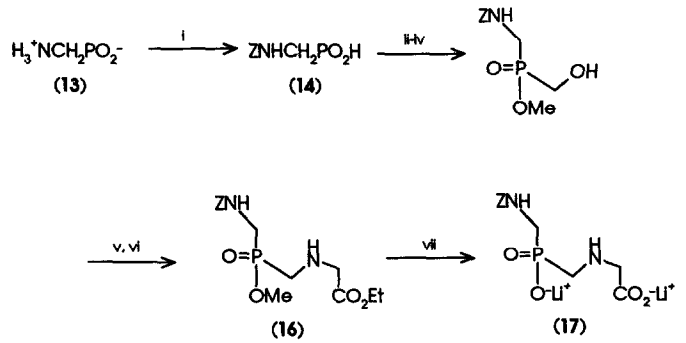
For (4-12)
Z=$PhCH_2OCO$

(3) Z = R = H
(4) R = H (Gly)
(5) R = Ph (D-Phegly)
(6) R = CH_2Ph (D-Phe)
(7) R = CH_2Ph (L-Phe)

(8) (D-Pro)
(9) (L-Pro)

(10) (D-thiazolidine)

(11) R = H (β-Ala)
(12) R = Ph (β-Phe-β-Ala)



Scheme 2: *Synthesis of 'expanded' TS analogue (17):* ***Reagents:*** *i. $PhCH_2OCOCl$, Na_2CO_3; ii. TMSCl, Et_3N; iii. CH_2O; iv. CH_2N_2; v. CF_3SO_2Cl, Et_3N; vi. $H_2NCH_2CO_2Et$; vii. LiOH, H_2O-CH_3CN.*

Table 1: *ID_{50} of phosphonamidates and clavulanic acid for a range of serine ß-lactamases*

	ID_{50} (μM)						
ß-lactamase	**TEM-1**	**P99**	***S.aureus* penicillinase**	**SHV-5**	**OXA-1**	***P.mirabilis* carbenicillinase**	**PSE-4**
Class (Bush)	2b	1	2a	2b′	2d	2c	2c
(4)	>100 (>100)	2.29	>100 (>100)	>100	30	>100	>100
(5)	>100 (>100)	4.16	>100 (>100)	>100	>100	>100	>100
(6)	>100 (>100)	3.80	>100 (>100)	>100	>100	>100	>100
(7)	50 (>100)	0.75	33 (>100)	>100	60	>100	15
(8)	>100	67	>100	NT	NT	NT	NT
(9)	>100	54	>100	NT	NT	NT	NT
(10)	>100	>100	>100	NT	NT	NT	NT
(11)	50 (>100)	0.87	25 (>100)	33	14	>100	>100
(12)	50 (>100)	0.87	35 (>100)	63	46	>100	83
(17)	>100	>100	>100	NT	NT	NT	NT
Clavulanic acid	0.315	310	0.38	0.013	3.4	0.51	0.235

Numbers in parenthesis = ID_{50}s determined with no pre-incubation of enzyme and inhibitor.

NT = Not tested.

Table 2: *Potentiation of amoxycillin by phosphonamidates and clavulanic acid*

Organism	ß-lactamase produced	MICs (µg/ml)								
		Amoxycillin	Clavulanic acid	Amoxycillin + 4µg/ml Clavulanic acid	MIC of Amoxycillin in Combination with 10µg/ml of each phosphonamidate (4)	(5)	(6)	(7)	(11)	(12)
Ent.cloacae N-1	P99	256	32	256	32	32	128	128	4	16
Staph.aureus MB9	Staph. penicillinase	256	32	0.12	64	64	64	64	32	8
E.coli E96 P^+	TEM-1	>256	32	16	>256	>256	>256	>256	>256	>256

Susceptibility Testing

MIC determinations were performed in liquid media in microdilution plates. All compounds were dissolved in Mueller-Hinton broth (Difco Laboratories). Amoxycillin (SmithKline Beecham) was then diluted serially in Mueller-Hinton broth followed by addition of inhibitor at a fixed concentration. Test compounds were used at 100, 10 and 1µg/ml. Clavulanic acid was included, at a lower concentration, as a reference standard. The MIC of inhibitor compounds alone was also determined.

Organisms were grown at 37°C for 18 hours in Mueller-Hinton broth and diluted to give a final count in each well of approximately 10^5cfu/ml. The total volume per well was 100µl.

Plates were incubated aerobically at 37°C for 18 hours. The MIC was recorded as the lowest concentration of amoxycillin to inhibit visible growth.

ID_{50} Determinations

ID_{50}s were performed against the following isolated enzymes (Bush classification):[18] *E.coli* TEM-1 (**2b**), *E.cloacae* P99 (**1**), *S.aureus* penicillinase (**2a**), *E.coli* SHV-5 (**2b'**), *E.coli* OXA-1 (**2d**), *P.mirabilis* carbenicillinase (**2c**), and *P.aeruginosa* PSE-4 (**2c**). Nitrocefin was employed as the reporter substrate, using the method described by Payne *et.al.*[19] ID_{50} values were determined following a 5 minute preincubation of enzyme and inhibitor. In addition, ID_{50}s for TEM-1 and *S.aureus* penicillinase were also determined without pre-incubation.

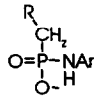
Results and Discussion

All the inhibitors showed their greatest activity against the P99 ß-lactamase; compounds (**7**), (**11**) and (**12**) were the most active inhibitors of P99 enzyme and also demonstrated the broadest spectrum of activity with ID_{50}s of less than, or equal to, 50µM for at least four of the enzymes tested. No inhibition of the TEM-1 or *S.aureus* ß-lactamases was observed without preincubation, suggesting the progressive nature of the inhibition (**Table 1**). Phosphinate (**17**) was inactive at the concentrations tested. The MICs of the phosphonamidates when tested alone, were >100µg/ml for all organisms. However, the presence of phosphonamidate (**11**) (10µg/ml) potentiated the antibacterial activity of amoxycillin against *E.cloacae* N-1 and *S.aureus* MB9 whole-cell bacteria by 64- and 8-fold, respectively (**Table 2**). Phosphonamidates (**4-7**) (10mg/ml) were less effective, reducing the MIC of amoxycillin for *E.cloacae* N-1

and *S.aureus* by (8-2)-fold and 4-fold, respectively. A concentration of 100μg/ml of (**11**) was required to reduce the MIC of amoxycillin for *E.coli* E96P by >2 fold. No other compound showed any potentiation of amoxycillin against this organism.

Mechanism of Inhibition

Pratt's work on the interactions of phosphonate monoesters (depsipeptide analogues) with serine ß-lactamases (notably the P99 class C enzyme)[20-23] has shown that enzyme inhibition occurs by a phosphonylation process rather than by transition state mimicry. This was proven by ^{31}PMR studies of



(**18**) R = ZNH

(**19**) R=H

inhibited enzyme, and by ^{14}C isotopic labelling.[21] More recent reports from this group comprise the demonstration that anilide phosphonamidates of types (**18**) and (**19**) are irreversible inhibitors of serine ß-lactamase, and a study of the pH profiles of inhibition stressed the requirement for leaving group protonation

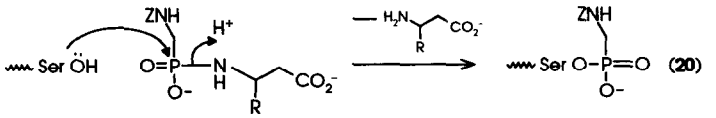


Figure 2: *Mechanism of inhibition by β-aminoacids (11) (R=H) and (12) (R=Ph)*

during the phosphonylation process.[13] The good inhibitory properties of phosphonamidates of ß-amino acids (**Table 2**) may arise as a consequence of the improved ease of protonation and nucleofugal properties of such leaving groups in comparison with their less basic, α-amino counterparts (**Figure 2**). Pratt's X-ray studies of a staphylococcal serine ß-lactamase inhibited by a methylphosphonate monoester are also in support of the general concept of such mechanisms.[23]

Our own ESMS studies of the P99 enzyme inhibited by *e.g.* phosphonamidates (**6**) and (**11**) show a similar mass increment corresponding to expulsion of the amino acid residue (*cf* **Figure 2**) [Mass=(M_{enz}+228) and (M_{enz}+229) amu, respectively; theoretical increment ΔM for the protonated form of (**20**)=227amu]. This is consistent with the operation of the foregoing active-site phosphonylation inhibition mechanism, both for α- and ß-amino acid C-terminal phosphonamidates.[24] Laws *et.al.* also demonstrated the phosphonylation of the P99 enzyme by the ethyl phosphonamidate ester corresponding to (**9**) using ESMS.[14]

Conclusions

- Phosphonamidates (**1**) have been shown to inhibit a range of serine ß-lactamases, with greatest activity against the *E.cloacae* P99 Class 1 enzyme.
- The series shows improved P-99 inhibitory activity over clavulanic acid.
- ß-Amino acid phosphonamidates show ~70-fold greater P99 inhibitory activity than compounds of type (**8**) and (**9**), which are similar to those previously reported.[14]
- Phosphonamidates have now been shown to potentiate the antibacterial activity of amoxycillin, which may be indicative of synergistic activity and cell penetration.

Acknowledgements

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24. A detailed account of these ESMS investigations will appear in our full paper. In most cases the experiments were set up to include the observation of free P99, together with inhibited enzyme, as an internal calibration for mass shift measurements.

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