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Antibacterial Activity and Mechanism of Action of Phosphonopeptides Based on Aminomethylphosphonic Acid

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Phosphonopeptides based on aminomethylphosphonic acid as the C-terminal residue linked to L-amino acids possessed antibacterial activity in vitro and in vivo. Analogs in this series were generally less potent than corresponding compounds based on L-1-aminoethylphosphonic acid such as alafosfalin (L-alanyl-L-1-aminoethylphosphonic acid). Significant differences in antibacterial spectra were observed. The mechanism of action involved active transport of the peptide mimetics into the bacterial cells, followed by intracellular release of high concentrations of aminomethylphosphonic acid which inhibited bacterial cell wall biosynthesis. Aminomethylphosphonic acid behaved as a mimetic of both D- and L-alanine and inhibited D-Ala-D-Ala synthetase (EC 6.3.2.4.), alanine racemase (EC 5.1.1.1.), and UDP-*N*-acetylmuramyl-L-alanine synthetase (EC 6.3.2.8.). The minimal inhibitory concentration of L-norvalyl-aminomethylphosphonic acid was essentially unaffected by the presence of D-alanine, whereas the activity of the corresponding L-norvalyl derivative of L-1-aminoethylphosphonic acid was markedly decreased. Substantial differences in the inhibitory and lytic activity of the L-norvalyl derivatives of aminomethylphosphonic and L-1-aminoethylphosphonic acids were also observed when these agents were combined with other inhibitors of bacterial cell wall biosynthesis.

Previous papers have described results of investigations on the antibacterial properties of phosphonopeptides (1–7). Alafosfalin [L-alanyl-L-1-aminoethylphosphonic acid; L-Ala-L-Ala(P)] has been selected for clinical studies in humans. Other related compounds incorporating L-Ala(P) are of interest for particular applications in therapy.

In these studies, phosphonopeptides with alternative aminoalkylphosphonic acid residues were investigated. Phosphonopeptides incorporating aminomethylphosphonic acid, Gly(P), were the only compounds with levels of antibacterial activity comparable with those of the L-Ala(P) series. In this paper, we investigated phosphonopeptides in which various L-amino acids are combined with Gly(P).

MATERIALS AND METHODS

Amino acid and peptide mimetics. The term amino acid mimetic as used in this paper refers to synthetic variants of conventional α-amino acids with the carboxyl group replaced by alternative acidic functions, usually the phosphoryl (P[O][OH]$_2$) group. Peptide mimetics incorporate residues of these amino acid mimetics. (All amino acids and amino acid mimetics described below are of the L-series unless otherwise indicated, and L is therefore generally omitted in the abbreviations.)

Chemicals. The phosphonopeptides described in this report were synthesized by the methods described previously (7).

Chemical characteristics of the new compounds described in this paper are as shown in Table 1. The symbols for higher unbranched amino acids (2-aminooctanoic acid [Aoc]) are those recommended by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry Commission on Biochemical Nomenclature (11). Ala-[^{14}C]Gly(P) and Nva-[^{14}C]Gly(P) were synthesized by R. J. Francis. All other radiochemicals were purchased from the Radiochemical Centre, Amersham, England. Chemicals were of analytical grade or the equivalent and were purchased from either BDH Ltd., Dorset, England, or Sigma Chemicals Ltd., London, England. The following antibiotics were obtained: D-cycloserine (Roche Products Ltd., Welwyn Garden City, England), cephalexin (Eli Lilly & Co., Basingstoke, England), and fosfomycin (Merck, Sharp & Dohme, Rahway, N.J.).

Bacteria. Organisms obtained as lyophilized preparations from the National Collection of Industrial Bacteria (NCIB), National Collection of Type Cultures (NCTC), and American Type Culture Collection (ATCC) are indicated by the appropriate accession numbers. Other bacteria were obtained as clinical isolates from within the United Kingdom. All strains were maintained on appropriate standard bacteriological media.

Determination of MIC and synergistic activity. Mini-

TABLE 1. Chemical characteristics of new phosphonodipeptides

Name	Molecular formula[a]	Melting point (°C)	$[\alpha]_D^{20b}$
Phosphonodipeptide *X*-Gly(P)			
X = natural L-amino acid or glycine			
X			
Arginine (Arg)	$C_7H_{18}N_5O_4P$	185	+21
α-Glutamic acid (Glu)	$C_6H_{13}N_2O_6P$	208–209	+60
Glycine (Gly)	$C_3H_9N_2O_4P$	252–254	NA[c]
Histidine (His)	$C_7H_{13}N_4O_4P$	~175	−78.9[d]
Methionine (Met)	$C_6H_{15}N_2O_4PS$	262–264	+55.6
Proline (Pro)	$C_6H_{13}N_2O_4P$	247–249	−29.7[d]
Serine (Ser)	$C_4H_{11}N_2O_5P$	236–238	+20.6[d]
Tyrosine (Tyr)	$C_{10}H_{15}N_2O_5P$	~200	+88.6[e]
X = L-H_2N-CH-[$(CH_2)_nH$]-CO (*n* = 2–8)			
2-Aminobutyryl (Abu)	$C_5H_{13}N_2O_4P$	263–265	+57.0
2-Aminopentanoyl (Nva)	$C_6H_{15}N_2O_4P$	273–275	+61.2
2-Aminohexanoyl (Nle)	$C_7H_{17}N_2O_4P$	272–274	+63.4
2-Aminoheptanyl (Ahe)	$C_8H_{19}N_2O_4P$	273–276	+61.0
2-Aminooctanoyl (Aoc)	$C_9H_{21}N_2O_4P$	265–270	+25.9[f]
2-Aminononanoyl (Ano)	$C_{10}H_{23}N_2O_4P$	265–270	+25.8[f]
2-Aminodecanoyl (Ade)	$C_{11}H_{25}N_2O_4P$	275	+19.2[g]

[a] Confirmed by C, H, and N analysis, which were in all cases within 0.5% of the theoretical value.
[b] Specific optical rotation (in degrees of rotation); 0.5% in H_2O unless otherwise stated.
[c] NA, Not applicable.
[d] C = 1% in H_2O.
[e] C = 1% in acetic acid.
[f] C = 0.5% in 1 N HCl.
[g] C = 0.5% in 0.1 N NaOH.

mum inhibitory concentrations (MICs) were determined by an agar incorporation method as described previously (7), using final concentrations of antimicrobial agents ranging from 0.007 to 128 μg/ml. The defined susceptibility medium, free from antagonists to small peptide mimetics, reported previously (2), or an improvement of this medium (5) which supported good growth of a wide range of organisms, was used throughout these studies.

Inhibitory synergy was measured by an agar checkerboard method. Ratios of antibacterial agents ranging from 4:1 to 1:4 for cephalexin-phosphonopeptide combinations and from 20:1 to 1:20 for D-cycloserine-phosphonopeptide combinations were tested by using the same final concentrations as for the MIC determinations. Average isobolograms were plotted by the method of Elion et al. (10) from the geometric means of fractional inhibitory concentration indexes calculated for individual strains.

In vivo activity. In vivo activity was studied in a mouse septicemia model by using groups of five female CFW or MF1 mice (17 to 19 g) infected intraperitoneally with 1.0-ml suspensions of overnight broth cultures diluted in mucin (2.5% [wt/vol] final concentration) to yield four to eight times the 99% lethal dose. Compounds were administered subcutaneously or orally at 1, 3, and 5 h postinfection in twofold dose steps as solutions freshly prepared in sterile physiological saline. The number of mice surviving for 7 days was used to calculate the 50% curative dose by the method of Weil (15). Each experiment included at least 20 infected untreated control animals, which usually died within 48 h. Surviving animals were not examined for persisting bacteria, but experiments were rejected if more than 10% of the untreated infected controls survived for 7 days.

Measurement of lysis by determining release of [^{3}H]uridine. Overnight cultures of *Proteus mirabilis* 502015 were diluted 10-fold with fresh defined medium and incubated at 37°C with 0.2 μM [^{3}H]uridine (5 Ci/mmol) for 3 h. Labeled cells were harvested by centrifugation and resuspended in fresh medium to a cell density of 10^8 bacteria per ml. After preincubation, the cultures were treated with drugs, and lysis was monitored by following leakage of tritiated material from the cells as previously described (4).

Transport and metabolism of phosphonopeptides. Early- to midexponential-phase cultures of *Escherichia coli* were harvested, washed, and suspended in 10 ml of defined medium or Davis minimal medium (9) at 37°C at a cell density of 1.5×10^8 to 3×10^8 bacteria per ml. After a 10-min preincubation to allow cells to recover, 100 μM Ala-[^{14}C]Gly(P) (7.6 mCi/mmol) was added to the 10-ml cultures. Samples (1 ml) were taken at intervals and centrifuged, and the cells were suspended in 0.1 ml of water. The cell contents were extracted from the 0.1-ml samples by heating them to 100°C for 5 min. After centrifugation in an Eppendorf microcentrifuge, 10-μl samples of the supernatants were applied to Whatman no. 1 filter paper (presoaked in 4% formic acid, pH 1.8), and Ala-Gly(P) was separated from its metabolites by high-voltage electrophoresis (100 V/cm) as described previously for alafosfalin (6).

Total uptake of phosphonopeptides was determined by using 5 ml-cultures as described above. Samples

(0.5 ml) were taken at intervals, and the cells were harvested by rapid filtration by using Whatman GF/C filter disks. The filters were washed twice with 5 ml of 0.8% (wt/vol) NaCl at 20°C and dried, and the radioactivity was determined by liquid scintillation counting. Transport studies were also carried out in the presence of D-alanine or peptides. Intracellular concentrations of phosphonopeptide related material were calculated by using a cell volume of 2.7 μl/mg (dry weight) of cells (16).

Incorporation of labeled precursor into whole-cell peptidoglycan and protein fractions. Cell wall and protein biosynthesis were measured in *E. coli* by following the incorporation of [*G*-2,6-*meso*-^{3}H]diaminopimelic acid and L-[*U*-^{14}C]valine into trichloroacetic acid-insoluble material as previously described (6). Peptidoglycan and protein biosynthesis in *Staphylococcus aureus* were both followed by measuring incorporation of L-[4,5-^{3}H]lysine and employing a selective pronase method to distinguish the fractions (8).

Isolation and characterization of cell wall precursor UDP-muramyl peptides in *S. aureus*. The contents of cells treated with Ala-Gly(P) were released by heating at 100°C for 5 min, the precipitated material was removed by centrifugation, and the *N*-acetylhexosamine content was determined (6). Nucleotide muropeptide precursors were separated from cell extracts by charcoal adsorption or ion-exchange chromatography. After elution, they were characterized by paper chromatography, electrophoresis, and amino acid analysis as previously described (6).

The standard muropeptide precursors uridine diphosphate-*N*-acetylmuramic acid (UDP-NAMA), UDP-NAMA-Ala, and UDP-NAMA-tri- and pentapeptides were obtained as previously described (12), and they were chemically identified by amino acid and hexosamine analysis and by Fourier-transform nuclear magnetic resonance.

Preparation and assay of other cell wall enzymes. D-Ala-D-Ala synthetase (EC 6.3.2.4) (*Streptococcus faecalis*), UDP-NAMA-L-Ala synthetase (EC 6.3.2.8) (*Staphylococcus aureus*), and alanine racemase (EC 5.1.1.1) (*E. coli*) were prepared and assayed by previously described methods (13, 14).

RESULTS

Antibacterial activity of *X*-Gly(P) phosphonodipeptides. Structure-activity relationships in the *X*-Gly(P) series were generally parallel to the corresponding *X*-Ala(P) compounds described previously (1, 5, 7). Where the N-terminal residue was a natural L-amino acid or glycine, the dipeptides containing Arg, Leu, or Met again showed the greatest antibacterial activity, whereas Gly-Gly(P) and Pro-Gly(P), which are particularly stable to peptide hydrolases (unpublished observations), were inactive at 128 μg/ml against all organisms tested (Table 2).

A series of *X*-Gly(P) compounds containing straight-chain aliphatic amino acids (X = L-$H_2NCH[(CH_2)_nH]$-CO [n = 0 to 8]) were highly potent, as for the corresponding *X*-Ala(P) case (5), with optimal activity again falling between n

TABLE 2. Antibacterial activities of phosphonodipeptides with the general formula *X*-Gly(P)

Strain	MIC (μg/ml) of the compounds with the following N-terminal α-amino acids (*X*) in the dipeptide *X*-Gly(P), where *X*[a] is:											
	Arg	Met	Leu	Tyr	Lys	Phe	His	Val	Ser	Ala	αGlu	Gly	Pro
Escherichia coli NCIB 8879	0.25	0.5	1	1	1	2	2	4	8	16	128	>128	>128
Klebsiella aerogenes 331001	0.5	0.25	0.25	0.5	1	1	2	1	4	4	2	>128	>128
Enterobacter 250002	4	4	2	8	8	8	16	16	64	32	64	>128	>128
Serratia marcescens ATCC 14756	8	8	2	4	32	8	8	8	64	32	32	>128	>128
Salmonella typhimurium 538003	4	4	2	8	8	8	16	16	32	64	64	>128	>128
Haemophilus influenzae NCTC 4560	64	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
Proteus mirabilis 502015	>128	128	128	>128	>128	>128	>128	>128	>128	>128	128	>128	>128
Proteus rettgeri	16	32	64	>128	>128	64		>128	>128	—	>128	>128	>128
Staphylococcus aureus NCIB 8625	8	2	4	8	16	4	8	2	16	8	>128	>128	>128
Streptococcus faecalis 585011	8	8	8	16	16	4	1	4	32	16	32	>128	>128
Bacillus subtilis NCTC 8236	4	2	0.5	2	16	2	2	1	8	—	128	>128	>128

[a] All amino acids are of the L-series, except for glycine.

TABLE 3. Antibacterial activities of phosphonodipeptides containing a homologous series of straight-chain aliphatic amino acids

Strain	MIC (μg/ml) of the compounds with the following N-terminal α-amino acid (*X*) in the dipeptide *X*-Gly(P), where *X* is:								
	Gly(0)[a]	Ala(1)	Abu(2)	Nva(3)	Nle(4)	Ahe(5)	Aoc(6)	Ano(7)	Ade(8)
Escherichia coli NCIB 8879	>128	16	1	0.5	0.5	0.5	0.25	1	8
Klebsiella aerogenes 331001	>128	4	0.25	0.12	0.5	0.25	0.12	0.5	4
Enterobacter 250002	>128	32	4	1	2	1	2	4	32
Serratia marcescens ATCC 14756	>128	32	2	1	1	1	1	2	16
Salmonella typhimurium 538003	>128	64	4	2	2	1	1	4	16
Haemophilus influenzae NCTC 4560	>128	>128	>128	128	128	>128	>128	>128	>128
Proteus mirabilis 502015	>128	>128	>128	64	64	64	32	64	>128
Providencia 504002	>128	128	4	2	2	2	1	2	16
Staphylococcus aureus NCIB 8625	>128	8	2	2	1	1	1	1	4
Streptococcus faecalis 585011	>128	16	8	8	4	4	8	8	32

[a] *X* = H_2N-CH(CH_2)$_n$H-CO; numbers within parentheses are values of *n*.

= 3 and *n* = 6, i.e., Nva-Gly(P) and Aoc-Gly(P) (Table 3). In contrast to the *X*-Ala(P) series, all compounds, including Nva-Gly(P), were inactive against *Pseudomonas aeruginosa* at 128 μg/ml. A series of sulfur-containing compounds of the general formula *X*-Gly(P) in which *X* = L-$H_2NCH[-CH_2S(CH_2)_mH]$-CO (*m* = 1 to 5) had no significant advantages in antibacterial activity in comparison to corresponding *X*-Gly(P) compounds containing straight-chain aliphatic amino acids.

Arg-Gly(P) and Nva-Gly(P) have been investigated for activity against clinical bacterial isolates and compared with alafosfalin and the corresponding Ala(P) analogs (Table 4). In general, the Gly(P)-containing dipeptides were less potent against most gram-negative bacteria. However, there were some significant differences in antibacterial spectra. Arg-Gly(P) was more effective than Arg-Ala(P) against *Proteus* isolates and against staphylococci. Nva-Gly(P) was more active than Nva-Ala(P) against staphylococci (Table 4). These in vitro results were reflected in vivo, using a mouse septicemia model (Table 5). Curative doses of Arg-Gly(P) and Nva-Gly(P) for infections with *Proteus mirabilis* 502075 and *Staphylococcus aureus* 561057 were lower than in the case of Ala(P) analogs.

Transport of phosphonodipeptides containing Gly(P) by *E. coli*. Transport of Nva-Gly(P) into *E. coli* NCIB 8879 occurred rapidly with the intracellular concentration of phosphonopeptide-derived material typically reaching 200 mM (Fig. 1). The larger part of this material was present as Gly(P). Ala-Gly(P) was transported at a significantly lower rate than Nva-Gly(P), whereas uptake of Gly(P) was almost undetect-

TABLE 4. Comparison of antibacterial activities of phosphonodipeptides *X*-Ala(P) with *X*-Gly(P) against clinical isolates

Species	*n*	Concn (μg/ml) of the following phosphonodipeptides which inhibit 50% of strains				
		Ala-Ala(P)	Arg-Ala(P)	Arg-Gly(P)	Nva-Ala(P)	Nva-Gly(P)
Escherichia coli	20	0.3	0.12	0.9	0.03	0.7
Klebsiella aerogenes	17	3.2	1.3	1.7	0.2	1.3
Enterobacter spp.	15	0.65	0.32	1	0.1	1
Citrobacter spp.	7	2.5	0.57	1.2	0.22	1.2
Serratia marcescens	12	1.6	0.58	1.2	0.17	0.9
Proteus mirabilis	20	>128	52	20	7	24
Proteus spp. (indole positive)	17	64	44	9	2.2	5
Salmonella typhimurium	19	2.7	0.66	4.3	0.17	2.7
Shigella spp.	16	0.04	0.01	0.33	<0.007	0.17
Haemophilus influenzae	7	22	23	70	4.3	29
Staphylococcus aureus	20	23	100	20	8	2.5
Staphylococcus epidermidis	20	8	32	20	3.5	2.8
Micrococcus spp.	20	32	>128	85	11	11
Streptococcus faecalis	19	0.7	0.7	5	0.35	1.4

TABLE 5. Activities of phosphonodipeptides *X*-Ala(P) and *X*-Gly(P) in the mouse septicemia model

Strain	Subcutaneous CD_{50} (mg/kg)[a]				
	Ala-Ala(P)	Arg-Ala(P)	Arg-Gly(P)	Nva-Ala(P)	Nva-Gly(P)
Escherichia coli 281007	7.1	2.3	6.8	1.2	18
Klebsiella aerogenes 331057	47	8.1	27	6.2	23
Proteus mirabilis 502075	≥1,000	353	101	154	54
Staphylococcus aureus 561057	≥1,000	≥1,000	92	>1,000	48
Streptococcus faecalis 585025	90	>500	97	28	56

[a] CD_{50}, 50% curative dose when given 1, 3, and 5 h after infection.

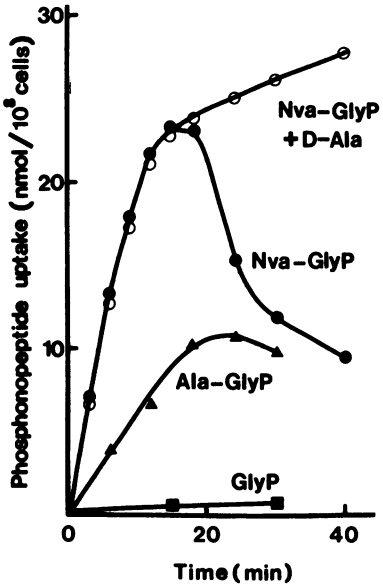

FIG. 1. Effect of D-alanine on Nva-Gly(P) uptake into *E. coli*. *E. coli* (2×10^8 bacteria per ml) in defined medium was incubated with 100 μM Nva-[^{14}C]Gly(P) (●) and in the same medium with 1 mM D-alanine (○). The uptake of 100 μM Ala-[^{14}C]Gly(P) (▲) or [^{14}C]Gly(P) (■) in the absence of D-alanine is shown for comparison.

able. After a 15-min incubation of *E. coli* with Nva-Gly(P), a decrease in turbidity occurred because of cell lysis, and radioactivity was released into the medium (Fig. 1). This rapid lytic phase was suppressed in the presence of D-alanine. Transport of Nva-Gly(P) into *E. coli* was inhibited by all L-di- and L-tripeptides but not by L-tetrapeptides, as previously observed for alafosfalin (6).

Effect of Ala-Gly(P) and Gly(P) on bacterial cell wall biosynthesis. Cell wall biosynthesis as measured by incorporation of precursor amino acids was inhibited in *E. coli* and in *Staphylococcus aureus* by Ala-Gly(P). In response to 100 μg of Ala-Gly(P) per ml, *Staphylococcus aureus* R2192 produced 6.6 nmol of hexosamine-positive material per ml of culture. Three putative intermediates were detected with electrophoretic mobilities that were consistent with the production of UDP-NAMA, UDP-NAMA-Ala, and muramyl tripeptide.

Electrophoresis of the cell contents of *E. coli* NCIB 8879 after Ala-[^{14}C]Gly(P) uptake revealed an additional metabolite tentatively identified as UDP-NAMA-Gly(P), possessing similar characteristics to metabolite M, which is formed in the presence of alafosfalin (6).

When the action of active metabolite Gly(P) on isolated enzymes was investigated, it was shown to be a competitive inhibitor of alanine racemase and UDP-NAMA-L-Ala synthetase, as was L-Ala(P) released from alafosfalin (Table 6). In addition, Gly(P) also inhibited D-Ala-D-Ala synthetase. This was presumably due to the absence of a chiral center on the carbon atom next to phosphorus which allows the Gly(P) to act as a mimetic of both L- and D-alanine.

Effect of D-alanine on the antibacterial activity

TABLE 6. Effect of Gly(P) on cell wall enzymes[a]

Enzyme	K_m (mM)	K_i (mM)		
		Gly(P)	L-Ala(P)[b]	D-Ala(P)[b]
Alanine racemase[c]	0.93	0.1	0.03	0.01
UDP-NAMA-L-Ala	0.3	2	3	Inactive[d]
D-Ala-D-Ala synthetase	0.5	7	Inactive[d]	0.6
	20	20		3

[a] K_m and K_i values were determined by double-reciprocal plots for reversible inhibition. The double values for D-Ala-D-Ala synthetase are for donor and acceptor sites, respectively (14).
[b] Data were taken from reference 6.
[c] Assayed for the L- to D-direction.
[d] Inactive at 10 mM.

TABLE 7. Effect of D-Ala on the antibacterial activity of Nva-Gly(P) and Nva-Ala(P)

Phosphonopeptide	Strain	MIC (μg/ml) in the presence of following concn (μg/ml) of D-Ala:			
		0	10	25	50
Nva-Gly(P)	*Escherichia coli* NCIB 8879	1	1	2	2
	Klebsiella aerogenes 331003	1	1	2	2
	Enterobacter 250002	1	1	2	2
	Serratia marcescens ATCC 14756	2	2	2	2
	Salmonella typhimurium 538003	2	2	4	8
	Shigella boydii 551007	0.25	0.25	1	1
	Proteus mirabilis 502015	32	32	32	32
	Staphylococcus aureus NCIB 8625	4	16	16	16
Nva-Ala(P)	*Escherichia coli* NCIB 8879	0.12	0.12	1	16
	Klebsiella aerogenes 331003	0.5	0.5	>128	>128
	Enterobacter 250002	0.25	0.25	64	>128
	Serratia marcescens ATCC 14756	1	1	4	16
	Salmonella typhimurium 538003	1	1	2	>128
	Shigella boydii 551007	0.015	0.03	>128	>128
	Proteus mirabilis 502015	32	32	128	>128
	Staphylococcus aureus NCIB 8625	16	32	32	32

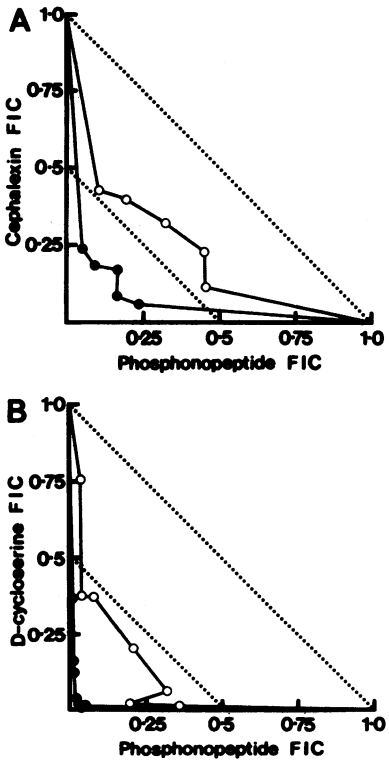

FIG. 2. (A) Average isobolograms of synergy between cephalexin and Nva-Ala(P) (●) or Nva-Gly(P) (○) against eight strains of *Staphylococcus aureus*. Combination MIC values were determined for ratios of agents ranging from 4:1 to 1:4. (B) Average isobolograms of synergy between D-cycloserine and Nva-Ala(P) (●) or Nva-Gly(P) (○) against eight strains of *Staphylococcus aureus*. Combination MIC values were determined for ratios of agents ranging from 20:1 to 1:20.

of Nva-Gly(P) and Nva-Ala(P). Alafosfalin induces an early lytic response in *E. coli* that is abolished by including D-alanine in the medium (6). A similar effect of D-alanine on lysis was observed when *E. coli* cells were treated with either Nva-Gly(P) or Nva-Ala(P) for 40 min (Fig. 1). However, D-alanine had very little effect on the antibacterial activity of Nva-Gly(P) measured in MIC determinations after 16 h of treatment. This was in contrast to a substantial reduction in the antibacterial activity of Nva-Ala(P) in the presence of D-alanine with most of the organisms examined (Table 7).

Synergy between Nva-Gly(P) and cell wall biosynthesis inhibitors. Nva-Gly(P) showed antibacterial synergy with cephalexin (Fig. 2A) and with D-cycloserine (Fig. 2B) in *Staphylococcus aureus* strains; this effect was more pronounced when Nva-Ala(P) replaced Nva-Gly(P) in the combinations.

Synergy was also observed in *Proteus mirabilis* by investigating lytic effects with other agents known to inhibit the early stages of bacterial cell wall biosynthesis. Nva-Gly(P) caused enhanced lysis of *Proteus mirabilis* when combined with fosfomycin, whereas this did not occur with Nva-Ala(P) (Fig. 3A). In contrast, when D-cycloserine was substituted for fosfomycin, lysis was more pronounced in combination with Nva-Ala(P) than with Nva-Gly(P) (Fig. 3B).

DISCUSSION

In general, the dipeptides of the Gly(P) series were less potent antibacterial agents than were the corresponding L-Ala(P) analogues. However, exceptions, such as Arg-Gly(P) for *Proteus* isolates and staphylococci and Nva-Gly(P) for

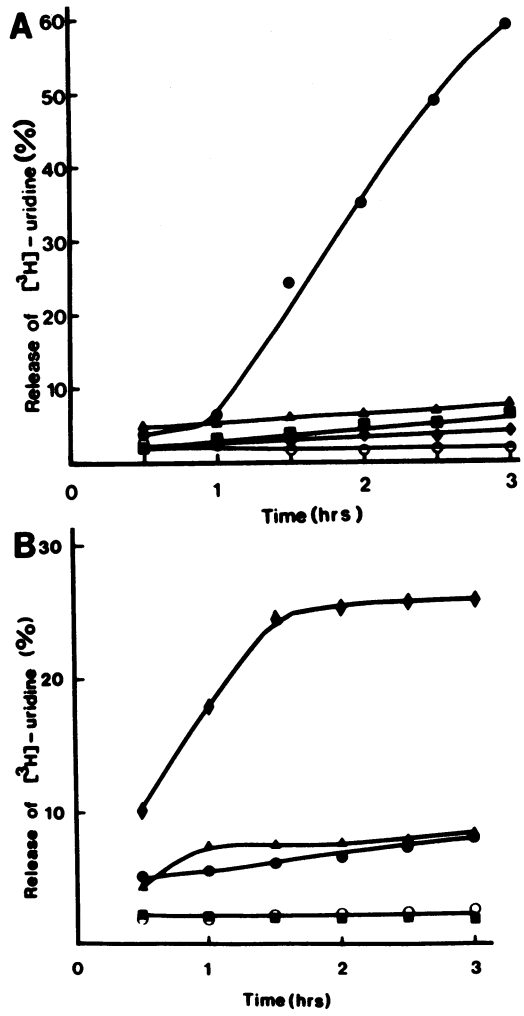

FIG. 3. (A) Lysis of *Proteus mirabilis* 502015 by fosfomycin in combination with either Nva-Gly(P) or Nva-Ala(P). Symbols: ▲, 5 μg of Nva-Gly(P) per ml; ■, 5 μg of fosfomycin per ml; ●, 5 μg of Nva-Gly(P) plus 5 μg of fosfomycin per ml; ○, 5 μg of Nva-Ala(P) per ml; ♦, 5 μg of Nva-Ala(P) plus 5 μg of fosfomycin per ml. (B) Lysis of *Proteus mirabilis* 502015 by D-cycloserine in combination with either Nva-Gly(P) or Nva-Ala(P). Symbols: ▲, 5 μg of Nva-Gly(P) per ml; ■, 5 μg of D-cycloserine per ml; ●, 5 μg of Nva-Gly(P) plus 5 μg of D-cycloserine per ml; ○, 5 μg of Nva-Ala(P) per ml; ♦, 5 μg of Nva-Ala(P) plus 5 μg of D-cycloserine per ml.

staphylococci, were notable. The mechanism of action of the Gly(P) dipeptides involved active transport, intracellular hydrolysis, and inhibition of bacterial cell wall biosynthesis and in several respects resembled that of the Ala(P) series. However, a difference in the response of gram-negative bacteria sensitive to Nva-Ala(P) and Nva-Gly(P) was noted. Whereas the initial lysis reaction to these agents was similar and was antagonized by D-Ala in both cases, the antimicrobial activity of Nva-Gly(P) alone was essentially unaffected by D-Ala upon longer term incubation.

It is possible that the early phase of lysis, which is common to both aminoalkylphosphonic acids, is due to the effect that they have in inhibiting alanine racemase, whereas the later phases are more influenced by the specific ability of Gly(P) to inhibit D-Ala-D-Ala synthetase. The results of experiments using combinations of Nva-Gly(P) with D-cycloserine are also consistent with this explanation. When D-cycloserine, acting as a potent inhibitor of D-Ala-D-Ala synthetase, was combined with Nva-Ala(P), cell lysis was substantially enhanced. These lytic effects were not observed when Nva-Gly(P) replaced Nva-Ala(P) in the combination.

In summary, the mechanism of action of dipeptides in the Gly(P) series differs from that of analogs based on Ala(P) in that the aminomethylphosphonic acid moiety can inhibit enzymes which utilize both D- and L-amino acids as substrates.

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