ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1980, p. 897-905
0066-4804/80/12-0897/09$02.00/0

Vol. 18, No. 6

Phosphonopeptide Antibacterial Agents Related to Alafosfalin: Design, Synthesis, and Structure-Activity Relationships

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Dipeptide variants of alafosfalin (L-alanyl-L-1-aminoethylphosphonic acid) with substantial differences in potency and antibacterial spectrum in vitro and in vivo have been synthesized. Certain dipeptides with alternatives to the L-alanyl residue had broader antibacterial spectra; activity against *Pseudomonas aeruginosa* was included. Some compounds had better in vivo activity than alafosfalin when introduced into infected rodents orally, but for the majority of the more active phosphonodipeptides, parenteral administration was more effective. Certain oligopeptides derived from the more active phosphonodipeptides possessed good in vitro activity against an extended range of organisms; they included *Haemophilus influenzae*, *Streptococcus faecalis*, and *Streptococcus pneumoniae*. The in vivo activity of some of these phosphono-oligopeptides was significantly greater than that of the parent dipeptide and correlated well with the in vitro results. This indicates that phosphono-oligopeptides exert part of their in vivo action directly, in addition to that arising from smaller peptides produced by peptidase cleavage.

Phosphonopeptides constitute a class of synthetic antibacterial peptide mimetics in which the C-terminal carboxyl group has been replaced by a phosphoryl [P(O)(OH)$_2$] function (1). These compounds are transported into bacteria by means of peptide permeases located in the bacterial cytoplasmic membrane. Within the cell they are cleaved enzymatically to liberate an aminoalkylphosphonic acid moiety which inhibits cell wall biosynthesis (3). In a previous study, phosphonopeptides containing L-amino acids linked to the L-1-aminoethylphosphonic acid Ala(P) [or less significantly, to aminomethylphosphonic acid, Gly(P)] as the acid terminal substituent were shown to form the most interesting series of compounds (4). On the basis of antibacterial activity in vitro and in vivo and stability to mammalian peptide hydrolase enzymes, alafosfalin [L-alanyl-L-1-aminoethylphosphonic acid; Ala-Ala(P)] was selected for detailed evaluation (1-4). (All amino acids and amino acid mimetics in this paper are of the L series, and L- is therefore omitted in abbreviations.)

Although alafosfalin had high levels of activity against certain members of the *Enterobacteriaceae*, including *Enterobacter* sp., *Escherichia coli*, *Klebsiella* sp., and *Shigella* sp., the antibacterial activity against *Proteus* and *Pseudomonas* spp. or streptococci (with the exception of *Streptococcus faecalis*) was modest (1, 2, 4). However, as the investigation of the mechanism of action of this new class of antibacterial agents developed, it appeared likely that phosphonopeptides with advantages over alafosfalin might be identified. This has been confirmed.

In our preliminary studies (4) we observed that certain phosphonodipeptides with different natural amino acids combined with Ala(P) showed very substantial differences in antibacterial activity. A detailed investigation of L-methionyl (Met)-Ala(P) established that the enhanced activity and broader antibacterial spectrum of this compound could be explained by more rapid transport into the bacteria than in the case of alafosfalin. Met-Ala(P) has not been developed further because it is more rapidly degraded by mammalian peptidases and is therefore less active in vivo than alafosfalin. This observation relating to Met-Ala(P) encouraged the examination of a wider range of dipeptides with the purpose of identifying phosphonopeptides with improved transport characteristics which retained, however, sufficient stability to mammalian peptidases to ensure good levels of antibacterial activity in vivo. The earlier studies (4) had also established that phosphono-oligopeptides displayed differences in their antibacterial spectrum in vitro from the corresponding phosphonodipeptides.

In this paper we describe investigations directed toward identifying novel phosphonopeptides with enhanced antibacterial activity both in vitro and in vivo.

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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.

Synthesis of phosphonopeptides. The phosphonopeptides described in this report were synthesized by the methods described previously (4).

Chemical characteristics of the new compounds described in this paper are as shown in Table 1. The symbols for N^2-alkylamino acids (*N*-ethylglycine = EtGly) and 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 (6).

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. All other bacteria were obtained as fresh clinical isolates from within the United Kingdom. *Enterobacter* sp. 250002 corresponds to strain 1398 in previous papers (2, 4), *E. coli* 281007 corresponds to strain 1346, *Klebsiella aerogenes* 331001 corresponds to strain KA1, *Proteus mirabilis* 502015 corresponds to strain 92, *Salmonella typhimurium* 538003 corresponds to strain S12, and *S. faecalis* 585011 corresponds to strain FS5. All strains were maintained on appropriate standard bacteriological media.

Determination of MIC. Minimum inhibitory concentrations (MICs) were determined by an agar incorporation method as described previously (4), but using final concentrations of agents ranging from 0.007 to 128 μg/ml. The defined susceptibility medium, free from antagonists to small peptide mimetics, reported in the previous communication (4) was used throughout these studies, except for the results shown in Table 8 for *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Streptococcus pyogenes*. In this instance the medium was modified as follows..

The broth (pH 6.9) contained the following: Ala, 0.025 g; Arg, 0.2 g; Asn, 0.1 g; Cys, 0.3 g; Cys_2, 0.2 g; Glu, 0.5 g; Gly, 0.2 g; His, 0.05 g; Ile, 0.3 g; Leu, 0.5 g; Lys hydrochloride, 0.2 g; Met, 0.025 g; Phe, 0.4 g; Pro, 0.2 g; Ser, 0.05 g; Thr, 0.2 g; Trp, 0.025 g; Tyr, 0.1 g; and Val, 0.5 g (all L-isomers); guanine, uracil, cytosine, and adenine, 0.01 g each; Na_2HPO_4, 4.5 g; KH_2PO_4, 1.5 g; NaCl, 2 g; $(NH_4)_2SO_4$, 1 g; $CaCl_2 \cdot 6H_2O$, 0.1 g; magnesium glycerophosphate $\cdot 1H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, and $CuSO_4 \cdot 5H_2O$, 0.001 g each; folic acid, biotin, calcium pantothenate, nicotinamide, pyridoxal hydrochloride, thiamine hydrochloride, riboflavine phosphate, and choline chloride, 0.005 g each; meso-inositol, 0.009 g; sodium pyruvate, 1 g; sodium citrate, 0.75 g; and D-glucose, 2 g; all dissolved in 1 liter of distilled water.

The agar medium was prepared as described above, but with the addition of 1% agar no. 1 (Oxoid Ltd., Basingstoke, England). All of the ingredients were specified by the manufacturer as AnalaR analytical reagent or chromatographically homogenous and were variously obtained from Sigma Chemical Co., St. Louis, Mo.; British Drug Houses, Poole, United Kingdom; or Hopkin and Williams, Chadwell Heath, United Kingdom.

The media were sterilized at 121°C for 15 min. When testing *S. pyogenes* and *S. pneumoniae* on agar, 7% defibrinated horse blood or 7% "chocolate" horse blood (for *H. influenzae*) was added. In broth culture studies, the addition of 5% horse serum was necessary for the streptococci, and a further addition of 2 ml/liter of a saturated solution of hemin in 0.5% ammonia (prefiltered on a GF/C filter to remove undissolved hemin, then filter sterilized) was necessary for *H. influenzae*.

Intracellular concentration of Ala(P) in bacteria. Unless otherwise stated, cell suspensions (50 ml; 3×10^8 bacteria per ml; late exponential phase) were incubated with 100 μM phosphonopeptide in the defined susceptibility medium (4) at 37°C for 15 min. The cells were harvested by centrifugation, washed with 0.9% (wt/vol) NaCl at 20°C, and suspended in 5 ml of water. The cell contents were extracted by heating to 100°C for 5 min, and 0.8-ml samples of the extract supernatant were analyzed with a JEOL amino acid analyzer as previously described (3).

Intercellular concentrations of Ala(P) were calculated from peak areas and corrected for an intracellular volume of 2.7 μl/mg (dry weight) of cells (16).

Transport studies with *S. pneumoniae* and *S. pyogenes* were carried out with the modifications described in the legend to Fig. 3.

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 (14). 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.

RESULTS

Antibacterial activity of phosphonodipeptides in vitro. In our earlier publications (1, 2, 4), the antibacterial activities of selected phosphonodipeptides in the series X-Ala(P) (X = L-amino acid) were reported. Further analogs have now been synthesized (Table 2) to extend this series to include the majority of the natural L-amino acids. Several compounds had greater antibacterial potency than alafosfalin. Like Met-Ala(P), which was reported previously, the Arg, Asp, Cit, α-Glu, and Leu analogs were weakly

TABLE 1. *Chemical characteristics of new phosphonopeptides*

Name	Molecular formula[a]	Melting point (°C)	$[\alpha]_D^{20}$[b]
Phosphonodipeptides: X-L-Ala(P)			
X = natural L-amino acid			
Arginine (Arg)	$C_8H_{20}N_5O_4P$[c]	ca. 190	−23.2
Asparagine (Asn)	$C_6H_{14}N_3O_5P$	265	−28.6
Aspartic acid (Asp)	$C_6H_{13}N_2O_6P$	ca. 220	−32.6
Citrulline (Cit)	$C_8H_{19}N_4O_5P$	259–260	−11.9
Cysteine (Cys)	$C_5H_{13}N_2O_4PS$	288–290	−32.2[d]
Cystine $(Cys)_2$	$C_8H_{18}N_3O_6PS_2$	205–209	−178[e]
Glutamic acid (Glu)	$C_7H_{15}N_2O_6P$	195–196	−8.4
Glutamine (Gln)	$C_7H_{16}N_3O_5P$	265	−12.2[f]
Histidine (His)	$C_8H_{15}N_4O_4P$	185	−120[g]
Isoleucine (Ile)	$C_8H_{19}N_2O_4P$	255–258	−7.8
Ornithine (Orn)	$C_7H_{18}N_3O_4P$[h]	242–244	−10.4
Serine (Ser)	$C_5H_{13}N_2O_5P$	250–251	−41.7
Threonine (Thr)	$C_6H_{15}N_2O_5P$	264–266	−34.5
Tryptophan (Trp)	$C_{13}H_{18}N_3O_4P$	232	+29.8[i]
Tyrosine (Tyr)	$C_{11}H_{17}N_2O_5P$	224	−24.4
X = L-H_2N-$CH(CH_2)_nH$-CO (n = 2–8)			
2-Aminobutyryl (Abu)[j]	$C_6H_{15}N_2O_4P$	296–298	−33.1[k]
2-Aminopentanoyl (Nva)	$C_7H_{17}N_2O_4P$	260–262	−19.5[l]
2-Aminohexanoyl (Nle)	$C_8H_{19}N_2O_4P$	253–255	−17.0[l]
2-Aminoheptanoyl (Ahe)	$C_9H_{21}N_2O_4P$	240–242	−15.8
2-Amino-octanoyl (Aoc)	$C_{10}H_{23}N_2O_4P$	255	−18.2[d]
2-Aminononanoyl (Ano)	$C_{11}H_{25}N_2O_4P$	252–257	−16.6[d]
2-Aminodecanoyl (Ade)	$C_{12}H_{27}N_2O_4P$	265	−16.5[d]
Phosphono-oligopeptides			
$(Ala)_5$-Ala(P)	$C_{17}H_{33}N_6O_8P$	320–322	−128[m]
$(Val)_2$-Ala-Ala(P)	$C_{15}H_{31}N_4O_6P$	307–309	−91.9[m]
$(Val)_2$-Nva-Ala(P)	$C_{17}H_{35}N_4O_6P$	280	−70.9[d]
Gly-$(Nva)_2$-Ala(P)	$C_{14}H_{29}N_4O_6P$	268–270	−83.1[d]
Sar-Ala-Ala(P)	$C_8H_{18}N_3O_5P$	245–246	−84.2
Sar-Nva-Ala(P)	$C_{10}H_{22}N_3O_5P$	258–260	−71.9[n]
Sar-$(Ala)_2$-Ala(P)	$C_{11}H_{23}N_4O_6P$	289–290	−121
Sar-$(Nva)_2$-Ala(P)	$C_{15}H_{31}N_4O_6P$	275–277	−83.5[g]
Sar-Ala-Tyr-Ala(P)	$C_{17}H_{27}N_4O_7P$	287–289	−53.4
Sar-Gly-$(Nva)_2$-Ala(P)	$C_{17}H_{34}N_5O_7P$	278–280	−85.8
Sar-$(Val)_2$-Nva-Ala(P)	$C_{20}H_{40}N_5O_7P$	292–294	−87.9[n]
(N-n-Pr)Gly-$(Ala)_2$-Ala(P)	$C_{13}H_{27}N_4O_6P$	260–262	−110[l]
(N-n-Pr)Gly-$(Nva)_2$-Ala(P)	$C_{17}H_{35}N_4O_6P$	289–290	−82.0[n]
(N-Allyl)Gly-$(Nva)_2$-Ala(P)	$C_{17}H_{33}N_4O_6P$	267–270	−73.4[n]
(N-Me)Val-$(Nva)_2$-Ala(P)	$C_{18}H_{37}N_4O_6P$	300–302	−51.1[n]
<Glu-$(Nva)_2$-Ala(P)	$C_{17}H_{31}N_4O_7P$	249–251	−81.1[n]

[a] Confirmed by C, H, and N analysis, which was in all cases within 0.5% of the theoretical value.
[b] Specific optical rotation (in degrees of rotation); c = 0.5%, H_2O unless otherwise stated.
[c] Analyzed as hemiacetate.
[d] Weight percent by volume (grams per 100 ml of solvent; c) = 0.5%, 1 N HCl.
[e] c = 0.25%, 2 N HCl.
[f] c = 0.6%, H_2O.
[g] c = 0.36%, H_2O.
[h] Analyzed as oxalate.
[i] $[\alpha]_{365}^{20}$
[j] Abbreviations as in the previous section.
[k] c = 1%, 1 N NaOH.
[l] c = 1%, H_2O.
[m] c = 0.5% in 1 N NaOH.
[n] c = 0.5% in trifluoroacetic acid.

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

Organism	MIC (μg/ml) of the compounds having the following N-terminal α-amino acids (X) in the dipeptide X-Ala(P), where X is:																		
	Met	Leu	Arg	Cit	His	Ala	Tyr	Ser	Asn	Ile	Trp	Cys_2	Thr	Gln	α-Glu	Asp	Orn	Cys
E. coli NCIB 8879	0.25	0.25	0.25	0.5	0.5	1	1	1	1	1	1	1	2	4	8	16	32	32
K. aerogenes 331001	0.12	0.12	<0.12	0.12	1	0.5	1	0.5	0.5	1	0.25	0.5	0.5	1	1	1	8	16
Enterobacter 250002	1	0.5	1	2	8	1	8	2	2	4	2	8	2	8	8	64	64	>128
S. marcescens ATCC 14756	2	2	2	4	16	8	16	8	8	16	4	8	16	8	8	16	128	>128
S. typhimurium 538003	8	8	32	16	>128	64	>128	32	64	128	16	64	64	128	16	16	>128	>128
H. influenzae NCTC 4560	16	32	128	64	128	64	128	16	128	32	128	128	4	128	128	>128	>128	>128
P. mirabilis 502015	32	32	128	64	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	16	32	>128	>128
S. faecalis 585011	1	2	16	4	4	2	4	16	32	4	64	4	4	8	8	8	>128	>128
S. aureus NCIB 8625	1	8	16	8	8	32	8	64	8	128	16	64	64	16	128	>128	>128	>128

active against *P. mirabilis* (Table 2). However, certain phosphonodipeptides containing branched-chain amino acids such as Ile (Table 2) or Val (4) were less potent antibacterial agents than alafosfalin.

As the Leu and Met analogs of X-Ala(P) had particularly interesting in vitro antibacterial properties, a series of compounds containing straight-chain aliphatic amino acids (X = L-H_2N-CH[$(CH_2)_nH$]-CO- [n = 0–8]) was synthesized. Optimum activity (Table 3) was observed with n = 3 and 4, i.e., Nva-Ala(P) and Nle-Ala(P); this included moderate levels against *Pseudomonas aeruginosa* (MICs, 32 and 64 μg/ml, respectively) (Table 3). Moreover, the Abu (n = 2) and Ahe (n = 5) analogs possessed similarly high levels of antibacterial activity, but were not active against *P. aeruginosa* (MIC, >128 μg/ml). Decreased activity was generally observed against most organisms with Ade-Ala(P) (n = 8), but for *S. typhimurium* or *P. mirabilis* this compound was as active as Nva-Ala(P) (Table 3).

Transport of phosphonodipeptides by bacteria. Phosphonodipeptides derived from the straight-chain aliphatic amino acids that were investigated delivered Ala(P) more effectively than alafosfalin into bacteria (Fig. 1) and were more potent. For the most part, there was a good correlation between the antibacterial potency of phosphonodipeptides and the rate of release of Ala(P) within the bacteria. The results for *E. coli* NCIB 8879 after treatment with various phosphonodipeptides are summarized in Fig. 2. In general, MICs were inversely proportional to the concentration of Ala(P) delivered, but His-Ala(P), Gly-Ala(P), and Val-Ala(P) were exceptions; in these cases another factor appears to be involved in the mechanism of action.

There was also a correlation between antibacterial activity and the rate of phosphonodipeptide transport in other organisms. In *P. mirabilis,* the intracellular liberation of Ala(P) from Arg-Ala(P), α-Glu-Ala(P), and Met-Ala(P) (analogs that have MICs of <128 μg/ml against this organism) was significantly higher than that from the less active analog, alafosfalin (Table 4). The α-Glu analog and phosphonodipeptides with aromatic amino acid residues were poorly transported by *P. aeruginosa* (Table 4); all were inactive (MIC, >128 μg/ml).

In the series of phosphonodipeptides X-Ala(P) in which X = L-H_2N-CH[$(CH_2)_nH$]-CO-, the rate of transport and subsequent Ala(P) liberation in *P. mirabilis* and *Serratia marcescens* occurred most rapidly at n = 2 to 8 (Fig. 1). Overall, there was a good correlation between transport parameters (Fig. 1) and antibacterial activity (Table 3).

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

Organism	MIC (μg/ml) of the compounds having the following N-terminal α-amino acids (X) in the dipeptide X-Ala(P), where X is:								
	Gly (0)[a]	Ala (1)	Abu (2)	Nva (3)	Nle (4)	Ahe (5)	Aoc (6)	Ano (7)	Ade (8)
E. coli NCIB 8879	8	1	0.25	0.03	0.06	0.03	0.015	0.12	0.25
K. aerogenes 331001	8	0.5	0.03	0.015	0.06	0.015	<0.007	0.12	2
Enterobacter 250002	64	1	0.5	0.25	1	0.06	0.06	0.25	4
S. marcescens ATCC 14756	>128	8	1	0.5	1	0.25	0.5	2	8
S. typhimurium 538003	>128	4	0.5	0.25	1	0.25	0.25	0.5	0.5
H. influenzae NCTC 4560	>128	32	4	4	8	32	32	128	128
P. mirabilis 502015	>128	>128	64	16	32	8	2	8	16
P. aeruginosa NCIB 8295	>128	>128	>128	32	64	>128	>128	>128	>128
S. faecalis 585011	>128	2	0.5	1	1	1	4	16	32
S. aureus NCIB 8625	>128	32	16	8	8	4	4	8	16

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

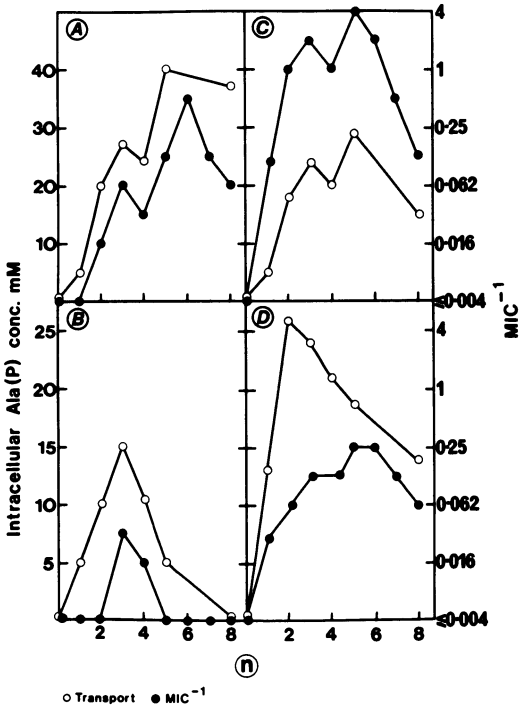

FIG. 1. *Intracellular delivery of Ala(P) and antibacterial action in several organisms for a homologous series of phosphonodipeptides containing straight-chain aliphatic L-amino acids. A, P. mirabilis 502015; B, P. aeruginosa NCIB 8295; C, S. marcescens ATCC 14756; D, S. aureus NCIB 8625. Intracellular Ala(P) levels arising from the phosphonodipeptides L-H_2N-CH(CH_2)$_n$H-CO-Ala(P) (values of n shown in abscissae) were determined by amino acid analysis as described in the text. MICs are expressed in micrograms per milliliter.*

In vivo antibacterial activity of phosphonodipeptides in the X-Ala(P) series. The in vivo antibacterial activities of representative phosphonodipeptides for oral and parenteral administration in an *E. coli* mouse septicemia model are summarized in Tables 5 and 6. Arg-Ala(P) was the only compound with considerably greater activity than alafosfalin when given by both routes. Nva-Ala(P) and, to a lesser extent, some related phosphonodipeptides were more active than alafosfalin when administered parenterally, but not when administered by the oral route.

Antibacterial activity of phosphono-oligopeptides in vitro. The earlier studies (4) established that phosphonotri- to hexapeptides had antibacterial properties in vitro that differed from related phosphonodipeptides with respect to both spectrum and potency. However, these differences were not expressed in vivo; in this case the potency and spectrum resembled those of the corresponding dipeptide, produced, presumably, by the action of mammalian peptidases. Consequently, oligopeptides with N-terminal sarcosyl, pyroglutamyl, and branched-chain amino acid residues such as valyl were investigated in vivo to determine their potential for longer life. Several phosphonotetrapeptides with N-terminal sarcosyl residues had high levels of activity against *H. influenzae* and *S. faecalis* in vitro (Table 7) in addition to the organisms that were sensitive to the related phosphonodipeptides (Tables 2 and 3). Replacement of this sarcosyl by pyroglutamyl, *N*-allylglycyl, or *N*-*n*-propylglycyl did not improve potency or spectrum. In general, related phosphonotetra- and pentapeptides have similar antibacterial properties in vitro (Table 7).

Unlike phosphonodi- and tripeptides, the corresponding tetra- and higher phosphono-oligopeptides that were investigated were active in vitro against *S. pneumoniae* and *S. pyogenes*, major gram-positive respiratory tract pathogens (Fig. 3 and Table 8). Moreover, it was found for *S. pneumoniae*, but not *S. pyogenes* (Table 8), that oligopeptides incorporating Nva-Ala(P) were more active than corresponding com-

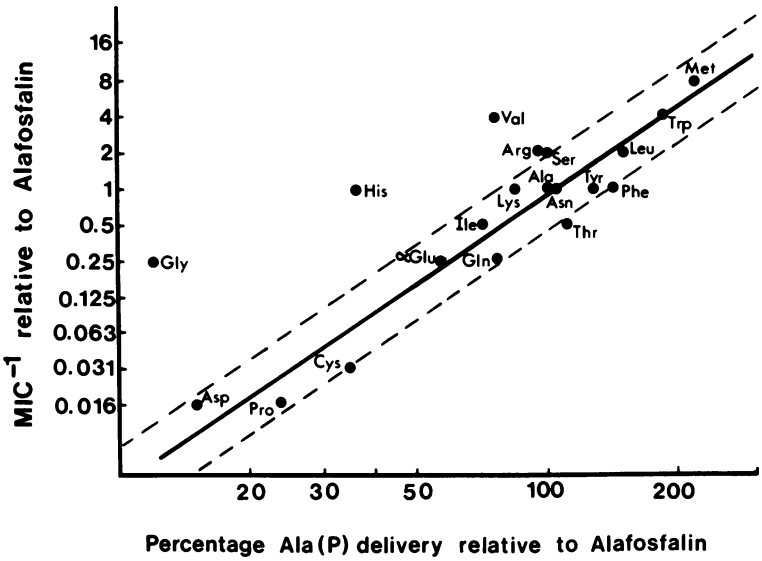

FIG. 2. *Relative intracellular delivery of Ala(P) and antibacterial action against E. coli NCIB 8879 for alafosfalin and a range of phosphonodipeptides. Antibacterial activity and intracellular Ala(P) levels arising from the dipeptides X-Ala(P) were determined as described in the text. Ala(P) levels are expressed as a percentage of that arising from the phosphonodipeptide alafosfalin [Ala-Ala(P)]. Lys, lysyl; Phe, phenylalanyl; Pro, prolyl.*

TABLE 4. *Intracellular concentration of Ala(P) arising from bacterial transport and intracellular hydrolysis of phosphonopeptides containing certain L-amino acids commonly found in proteins*

Organism	Intracellular Ala(P) concn (mM) arising from the dipeptide X-Ala(P) where X is					
	Ala	Arg	α-Glu	Met	Trp	Tyr
P. mirabilis 502015	5	22	19	24	5	2.5
P. aeruginosa NCIB 8295	5	4	2	8	0	0

TABLE 5. *Activities of phosphonopeptides [X-Ala(P)] containing a common L-amino acid (X) relative to alafosfalin against E. coli 281007 in the mouse septicemia model*

X	Relative activity [CD_{50} of alafosfalin/CD_{50} of X-Ala(P)][a]	
	Subcutaneous route	Oral route
Tyr	0.6	1.1
Ser	0.7	1.4
Ala	1.0	1.0
Val	1.1	0.4
Cit	1.4	1.7
Met	1.7	0.5
Leu	3.0	0.5
Arg	5.9	5.3

[a] CD_{50} (50% curative dose) values were determined in milligrams per kilogram.

TABLE 6. *Activities of phosphonodipeptides [X-Ala(P)] containing a homologous series of straight-chain aliphatic L-amino acids (X) relative to alafosfalin against E. coli 281007 in the mouse septicemia model*

X	Relative activity (CD_{50} of alafosfalin/CD_{50} of analog)[a]	
	Subcutaneous route	Oral route
Ala	1.0	1.0
Abu	5.6	1.5
Nva	9.0	1.0
Nle	5.9	1.3
Ahe	2.6	1.3
Aoc	5.2	1.7
Ano	<0.4	<0.4
Ade	1.0	<0.25

[a] CD_{50} (50% curative dose) values were determined in milligrams per kilogram.

pounds with alafosfalin residues; this may be attributed to the known limitation of this organism in cleavages of alanyl peptides (7) (Table 9). The phosphono-oligopeptides were, in general, more active against *H. influenzae* and *S. faecalis* than were the related phosphonodipeptides (Tables 7 and 8).

Antibacterial activity of phosphono-oligopeptides in vivo. Several of the N-terminal-substituted phosphono-oligopeptides possessed

TABLE 7. *Antibacterial activity of phosphono-oligopeptides Y-(X)$_n$-Ala(P)*

Organism	MIC (μg/ml) of Y-(X)$_n$-Ala(P) where N-terminal amino acid or peptide Y-(X)$_n$ is[a]:													
	Ala	(Ala)$_2$	(Ala)$_3$	(Val)$_2$ Ala	(Val)$_2$ Nva	Gly (Nva)$_2$	SarAla	SarNva	Sar(Ala)$_2$	Sar(Nva)$_2$	PrGly (Nva)$_2$	AlGly (Nva)$_2$	SarGly (Nva)$_2$	<Glu (Nva)$_2$
E. coli NCIB 8879	0.5	0.5	0.12	0.5	0.03	0.12	1	0.5	0.03	0.25	0.5	0.5	0.25	0.03
K. aerogenes 331001	0.25	0.12	0.5		0.06	0.25	4	0.5	0.12	0.5	0.25	0.25	0.12	0.12
Enterobacter 250002	4	8		8	0.25	1	32	0.5	2	1	4	4	0.5	2
S. faecalis 585011	2	0.06	0.25	0.5	0.5	0.5	4	0.5	0.06	0.12	0.25	0.06	0.12	0.25
S. aureus NCIB 8625	32	8	32	32	8	64	>128	>128	>128	>128	>128	>128	4	4
S. marcescens ATCC 14756	8	4	4	16	2	2	>128	64	1	8	32	16	8	4
S. typhimurium 538003	4	32	128	>128	1	16	>128	64	>128	8	32	8	4	2
H. influenzae NCTC 4560	32	<0.12	<0.12	<0.12	0.5	0.5	>128	128	0.03	0.03	0.06	0.03	0.06	0.06

[a] Abbreviations: X = L-amino acid or Gly; Y = X, N-alkyl-glycyl or <Glu (pyroglutamyl); n = 0–3; Sar, *N*-methylglycyl; PrGly, *N*-*n*-propylglycyl; AlGly, *N*-allylglycyl.

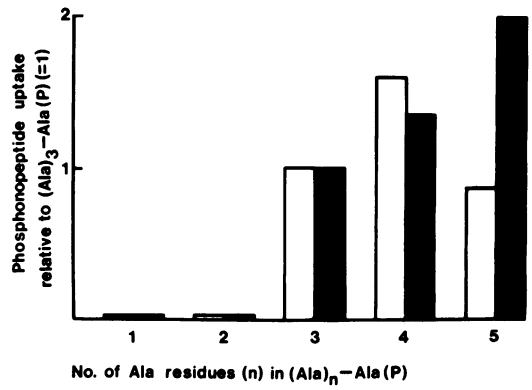

FIG. 3. *Transport of phosphonopeptides of the general formula (Ala)$_n$-Ala(P) (n = 1–5) by S. pneumoniae NCTC 7465 (solid bars) and S. pyogenes 588044 (open bars). Stationary bacterial cultures were suspended in defined medium (4) plus 2.5% (vol/vol) horse serum at 2.5 × 10^9 bacteria per ml. The uptake of phosphonopeptides (100 μM final concentration) was determined by amino acid analysis as described in the text.*

high in vivo activity approaching that of Nva-Ala(P) against a typical *E. coli* infection. Moreover, they were more active than the corresponding phosphonodipeptide against *S. faecalis* in the mouse septicemia model (Table 10). This indicated that the activity of the oligopeptide was not due alone to the dipeptide produced by peptidase cleavage in vivo. In the case of *S. pyogenes* (Table 10) potency was low and somewhat variable, whereas the phosphonodipeptide series had no action against this organism. *S. pneumoniae* was susceptible to certain phosphono-oligopeptides; strains 6431 and 6432 were inhibited by Sar-Nva-Nva-Ala(P) and pyroglutamyl (<Glu)-Nva-Nva-Ala(P) (50% curative doses for parenteral administration, 86 mg/kg and 67 mg/kg, respectively; we are indebted to R. Cleeland, Research Division, Hoffmann-La Roche, Inc., Nutley, N.J., for these results).

DISCUSSION

The investigations which were described in our earlier publications (1, 3) provided evidence to suggest that the action of phosphonopeptides on bacteria in vitro took place in three stages: (i) active transport from the medium into the bacterial cell, facilitated by cytoplasmic membrane permeases; (ii) intracellular cleavage to release the alanine mimetic, Ala(P); and (iii) interference with cell wall biosynthesis by the alanine mimetic through inhibition of alanine racemase and of uridine diphosphate-*N*-acetylmuramyl-alanine synthetase.

The variation in potency of various phosphon-

TABLE 8. *In vitro antibacterial activity of phosphono-oligopeptides Y-(X)$_n$-Ala(P) against respiratory tract pathogens*

Organism	No. of strains tested	MIC (μg/ml) of Y-(X)$_n$-Ala(P) where N-terminal amino acid or peptide Y-(X)$_n$ is[a]:							
		Ala	Nva	Sar(Ala)$_2$	PrGly (Ala)$_2$	SarAlaTyr	Sar(Nva)$_2$	Gly(Nva)$_2$	SarGly (Nva)$_2$
H. influenzae	6	32	4	0.03	0.02	0.04	0.015	0.6	0.13
S. pneumoniae	7	>128	>128	18	48	0.23	0.15	4	1.8
S. pyogenes	6	>128	>128	29	16	29	6.3	16	8

[a] Geometric mean MIC based on number of strains tested. X, Y, and *n* are as in Table 7.

TABLE 9. *Comparison of intracellular metabolism of selected phosphonopeptides by S. pneumoniae* NCTC 7465

Y-(X)$_n$-Ala(P)[a]	Metabolites (nmol/ml of cell extract)[b]				
	Ala(P)	Alafosfalin	Nva-Ala(P)	Total[c]	% as Ala(P)
Sar(Ala)$_2$Ala(P)	15	124		139	11
PrGly(Ala)$_2$Ala(P)	28	209		237	12
(Val)$_2$AlaAla(P)	79	557		636	12
Gly(Nva)$_2$Ala(P)	236		276	512	46
SarGly(Nva)$_2$Ala(P)	215		241	456	47

[a] Y, X, and *n* are as defined in Table 7.

[b] Determined as described in the text after incubation of *S. pneumoniae* NCTC 7465 at a cell density of 2.6 × 10^9 bacteria per ml with 100 μM phosphonopeptide for 60 min in defined medium (4) plus 2.5% (vol/vol) horse serum.

[c] The concentration of intact phosphonopeptides with *N*-alkylated N-terminal amino acids has not been taken into account in these calculations.

TABLE 10. *Activity of phosphonodipeptides and phosphono-oligopeptides in the mouse septicemia model*

Organism	CD_{50} (mg/kg) of Y-(X)$_n$-Ala(P) where N-terminal amino acid or peptide Y-(X)$_n$ is[a]:							
	Ala	Nva	Sar(Nva)$_2$	SarGly (Nva)$_2$	Sar(Val)$_2$ Nva	PrGly (Nva)$_2$	MeVal (Nva)$_2$[b]	<Glu(Nva)$_2$
E. coli 281007	6.5	0.8	1	4.1	12.4	<1.8	5.4	7.7
S. faecalis 585025	115	31	1.4	22	4.1	4.1	14	5.8
S. pyogenes 588061	>200	>200	64	>200	65	54	131	>200

[a] CD_{50} (50% curative dose) when given subcutaneously 1, 3, and 5 h after infection. X, Y, and *n* are as in Table 7.

[b] MeVal, *N*-Methylvalyl.

opeptides in vitro may be attributed to differences in their action on different bacteria at one or more of these stages.

It has become clear from our investigations (1, 3, 4) and from the work of others (8–12) that active transport of peptides into bacteria varies greatly with the organism and the composition of the peptides. This largely accounts for the differences in potency observed with phosphonopeptides. In the case of *E. coli* NCIB 8879, Fig. 2 summarizes data for 20 phosphonodipeptides. The cases of Gly-Ala(P), His-Ala(P), and Val-Ala(P) are exceptional in that the potency is greater than expected from the intracellular concentration of Ala(P) observed. Although these instances have not been studied further in detail, the result could be explained by a significant difference from alafosfalin in the rate of intracellular cleavage of these dipeptide mimetics.

The incorporation of unnatural amino acid residues with various lengths of aliphatic side chain into phosphonodipeptides was investigated after the observation that high antibacterial activity was associated with Met-Ala(P) and Leu-Ala(P). Again, high intracellular delivery of Ala(P) accounted for the antibacterial potency; there was no indication that the α-amino carboxylic acid itself was contributing significantly to the antibacterial activity. Although some antibacterial action through a surfacè-active effect occurs with compounds such as lauryl sulfate (5), the fact that Ade-Ala(P) had antibacterial

activity equivalent to that of Nva-Ala(P) against *P. mirabilis* and comparable activity against other organisms (Table 3) suggested that this was not relevant to the case of phosphonodipeptides.

Phosphonodipeptides were only transported slowly by *P. aeruginosa*. This may reflect a strict structural requirement of the peptide permeases of this organism or, alternatively, the poor penetration of the outer membrane which has been observed for many compounds, including β-lactam antibiotics (13, 15). In the case of Nva-Ala(P), which inhibited the growth of *P. aeruginosa* NCIB 8295 (MIC, 32 μg/ml), the rate of transport and intracellular Ala(P) delivery accounted for the observed activity.

It was apparent from this and our earlier studies that phosphono-oligopeptides displayed significant differences in in vitro activity from related phosphonodipeptides. *S. pneumoniae* and *S. pyogenes* are nonsusceptible to the dipeptides, but oligopeptides with four or more residues, which are transported by these bacteria, inhibit these organisms (Fig. 3 and Table 8). It is interesting that the ability of certain natural oligopeptides to display strepogenin activity (17) and growth-promoting properties (18) for streptococci may well reflect the active transport of such peptides by permeases.

The phosphono-oligopeptides that were investigated for antibacterial activity against a range of organisms (Table 7), including important respiratory tract pathogens (Table 8), included compounds with N-terminal residues such as sarcosyl and pyroglutamyl which were intended to facilitate their use as in vivo agents. Although there was some reduction in antibacterial potency when these residues were incorporated rather than those of simple α-amino carboxylic acids, good levels of activity against many species were retained. The investigation in vivo confirmed that compounds such as Sar-Nva-Nva-Ala(P) had interesting potential as in vivo antibacterial agents (Table 10).

In conclusion, these investigations have identified novel phosphonodipeptides and oligopeptides with interesting antibacterial activity in vivo. On the basis of this evidence, some of these compounds offer prospects for therapeutic use, either alone or in combination with other antibacterial agents that inhibit cell wall biosynthesis. Our studies on synergism with β-lactam antibiotics will be reported shortly.

ACKNOWLEDGMENTS

The advice of L. J. Nisbet and D. Westmacott regarding the in vitro testing of these compounds is gratefully acknowledged.

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