Antibiotics

VI

Modes and Mechanisms of Microbial Growth Inhibitors

Edited by F. E. Hahn

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Modes and Mechanisms of Microbial Growth Inhibitors

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With 127 Figures

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Professor FRED E. HAHN, Ph.D.
Division of Communicable Diseases and Immunology
Walter Reed Army Institute of Research
Washington, D.C. 20307, USA

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Preface

It is not certain that the editors of *Antibiotics I* (1967), Drs. GOTTLIEB and SHAW, fully realized that they were laying the foundation for an entire series of which we present here Vol. VI. For some time to come, this will be the last volume of the *Antibiotics* series.

There are several reasons for this. Firstly, the discovery of medicinally useful antibiotics has leveled off, because the number of microbiological products with antimicrobial properties is not infinite. In 1972 some 2500 antibiotic substances were known, of which approximately one per cent are clinically useful. Further search for antibiotics has led to increasing frequency of rediscoveries and drastically decreasing frequency of discoveries of new antibiotics. As the search for antibiotics with a standard methodology in conventional ecological niches has exhausted itself, there is a paucity of new and interesting substances on which to undertake modes/mechanisms of action studies.

Secondly, the mechanism of action field has come of age and its results are now academic knowledge. This also holds true for synthetic chemotherapeutic drugs and becomes the case rapidly for toxic substances with anti-eukaryotic action. The study of mechanisms of action was undertaken for two reasons: one was the basic scientific desire to know how antimicrobial substances interfered with microbial biochemistry; the second one was the hope that such information would be useful in the premeditated design of synthetic antimicrobials. The academic part of the undertaking has been largely satisfied through research of the past three decades. But the practical promise in terms of premeditated drug design has not been fulfilled to the extent originally anticipated. One instance of successful drug design from biochemical principles is that of alafosfalin which is the subject of one of the chapters of this book.

As a consequence of the exhaustion of objects of study and of the near completion of the investigation of known compounds, the source literature on mechanisms of action has decreased to comparatively few new publications. Mechanisms of action are no longer prominent sections of the programs of scientific meetings, dedicated to chemotherapy. The field that was in the forefront of science in the 1950's is now fighting a rearguard action, filling in details of mechanistic knowledge that, in principle, is well established.

This Preface is not the proper place to discuss ways and means by which the hiatus in new drug discovery may be overcome. This editor is confident that new search and research concepts might produce a new wave of discovery or development of chemotherapeutic drugs. If and when this happens, the time may come for yet another volume of Antibiotics.

A few drugs have fallen by the wayside and have not been treated recently or at all in the Antibiotics series, mostly because the scientific authorities on these drugs were not prepared to undergo the labor of writing systematic contributions. This is true, foremost, for the semisynthetic penicillins and cephalosporins despite their great medical importance. Antibiotics, therefore, remains only as complete as the joint efforts of authors and editors could render it, but it is not a scientific dictionary or encyclopedia and, for practical reasons, cannot be expected to be developed to this ideal state of completion.

The usefulness of this volume and of its predecessors lies in the fact that a very large volume of source literature has been considered and critically reviewed in order to make in-depth information on mechanisms of action of chemotherapeutic drugs easily available. This type of treatment broadly exceeds the descriptions of drug actions in academic textbooks. The editor hopes that Vol. VI will be as well received by the scientific community as have been the preceding volumes of this series.

The publisher has supported the assemblage of this book with unflinching interest and also should be thanked for the production of such a handsome and well-appointed book.

Washington, D.C., 1982 FRED E. HAHN

Contributors

CADMAN, ED, Department of Internal Medicine, Section of Medical Oncology, Yale University School of Medicine, New Haven, P.O. Box 3333, CT 06510, USA

CANONICO, PETER G., U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Frederick, MD 21701, USA

CARRASCO, LUIS, Departamento de Microbiología, Centro de Biología Molecular, Universidad Autónoma de Madrid, Canto Blanco, Madrid 34, Spain

COCITO, C., Department of Microbiology and Genetics, Institute of Cellular and Molecular Pathology, University of Louvain, Medical School, B 1200 Brussels, Belgium

CREASEY, WILLIAM, A., E.R. Squibb & Sons, Inc., The Squibb Institute for Medical Research, P.O. Box 4000, Princeton, NJ 08540, USA

EDWARDS, DAVID, I., North East London Polytechnic, Department of Paramedical Sciences, Romford Road, London E15 4LZ, England

FISCHER, E., Universität Tübingen, Institut für Biologie II, Auf der Morgenstelle 28, D-7400 Tübingen 1, FRG

GUPTA, RADHEY S., Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5, Canada

HAHN, FRED E., Walter Reed Army Institute of Research, Washington, DC 20307, USA

HASSALL, CEDRIC H., Roche Products Ltd., P.O. Box 8, Welwyn Garden City, Hertfordshire AL7 3AY, England

JIMÉNEZ, A., Centro de Biología Molecular, Instituto de Bioquímica de Macromoléculas, Universidad Autónoma de Madrid, Madrid 34, Spain

JONÁK, J., Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Flemingovo nám. 2, 166 10 Praha 6, CSSR

KNOWLES, JEREMY R., Department of Chemistry, Harvard University, 12, Oxford Street, Cambridge, MA 02138, USA

KOMIYAMA, KANKI, The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan

REUSSER, FRITZ, The Upjohn Company, Kalamazoo, MI 49001, USA

RYCHLÍK, IVAN, Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Flemingovo nám. 2, 166 10 Praha 6, CSSR

TANAKA, NOBUO, Institute of Applied Microbiology, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

TKACZ, JAN S., Waksman Institute of Microbiology, Rutgers, The State University of New Jersey, P.O. Box 759, Piscataway, NJ 08854, USA

UMEZAWA, I., The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan

VÁZQUEZ, DAVID, Centro de Biología Molecular, Instituto de Bioquímica de Macromoléculas, Universidad Autónoma de Madrid, Canto Blanco, Madrid 34, Spain

WOLF, HEINZ, Universität Tübingen, Institut für Biologie II, Auf der Morgenstelle 28, D-7400 Tübingen 1, FRG

WOLFE, ALAN D., Department of Biological Chemistry, Walter Reed Army Institute of Research, Washington, DC 20307, USA

WYSOR, MICHAEL S., Department of Parasitology, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20307, USA

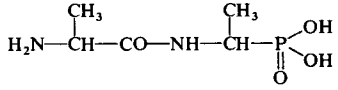
Contents

Alafosfalin (Ro 03-7008, Alaphosphin)

C.H. HASSALL

This new antibacterial agent is active in vitro and in vivo against a range of organisms; it is particularly effective against Gram-negative bacteria. Alafosfalin is a phosphonodipeptide (L-alanyl-L-1-aminoethylphosphonic acid, I) which is readily synthesized from simple starting materials. This compound was selected from a group designed as inhibitors of bacterial cell wall biosynthesis (ALLEN et al., 1978).

During the last 4 years the properties and mechanism of action of alafosfalin have been reported. This review provides an outline of the current status of knowledge of this new agent.



L-Ala L-Ala(P)

(I)
Alafosfalin

Toxicology and Safety Evaluation

Studies in the mouse, rat, and baboon (JACKSON and PARKES, unpublished) have established that alafosfalin has a low order of toxicity for animals. The lethal dose of the drug, given orally, is in excess of 10 g/kg for mice and rats and 4 g/kg for baboons. No adverse effects on the general condition of the animals were observed in 90-day studies with the rat (sc and oral) and the baboon (oral) at several dose levels up to 800 mg/kg and 2000 mg/kg, respectively. A small reduction in erythrocyte count was detected in rats after 4 weeks of treatment (sc) with 800 mg/kg and after daily dosing (po) with 4000 mg/kg for 8 weeks or 800 mg/kg for 13 weeks. It was shown with baboons after daily sc or im injection of 400 mg/kg for 8 weeks but not after po dosing of 2000 mg/kg for 13 weeks. The effect never exceeded 15% reduction in erythrocyte count, haematocrit or haemoglobin level. It was readily reversible when dosing was stopped. Similar small effects have been observed with other antibacterial agents (CAPEL-EDWARDS et al., 1977).

These and additional fertility, reproductive, and pharmacokinetic studies in animals (ALLEN et al., 1979a) have provided a good basis for undertaking trials in man.

Antibacterial Studies In Vitro

Spectrum. Alafosfalin is active in vitro against a wide range of bacteria, as illustrated in Table 1. The in vitro activity is significantly reduced in the presence of casein hydrolysate and peptones. The optimum pH for antibacterial activity is 5.5 and it is markedly less at pH 7.5. The antibacterial potency is significantly reduced when the size of the bacterial inoculum is increased from the standard 10^4 to 10^7 colony forming units/plate (ALLEN et al., 1979b). Comparison with commonly used antibiotics, including ampicillin, cephalexin, tetracycline, and cotrimoxazole (ALLEN et al., 1979b), using a range of clinical isolates, indicated that alafosfalin was superior to these agents against common Gram-negative bacteria, in vitro, except for *Proteus* and *Pseudomonas*. However, in general, it was less active against Gram-positive bacteria.

There have been further studies with clinical isolates in Japan (MARUYAMA et al., 1979).

Resistance. Against Gram-negative bacteria from clinical isolates, resistance to alafosfalin was found to be low (0–5%) but it was somewhat higher for Gram-positive genera (Table 2).

There was little evidence of cross-resistance between alafosfalin and other antibiotics; these included penicillins and related β-lactams that are effective in inhibiting bacterial cell-wall biosynthesis. The development of resistance to alafosfalin has been studied in vitro. In a typical experiment using *E. coli* the

Table 1. Antibacterial Spectrum of Alafosfalin

Organism	ID_{50} broth	MIC agar		
	Ro03-7008	Ro03-7008	PenG	Amp
E. coli 17	0.05	0.06	16	2
E. coli amp R		0.06	>128	>128
K. aerogenes A1	0.05	0.05	32	32
Str. faecalis 5	1.0	1	1.25	1
Micrococcus 7526	1.0	2	<0.12	<0.12
Entrobacter OG2	1.3	4	>128	>128
Ser. marcescens M42	1.4	4	64	8
S. albus 7	1.6	4	<0.12	<0.12
Sal. typhimurium TS6	1.7	8	4	0.5
Citrobacter FT2	2.4	2	16	4
Providence R2	2.6	4	16	32
S. aureus Schoch	3.4	8	<0.12	<0.12
Shigella flexneri		0.25	8	1
H. influenzae H11		16	0.5	0.5
N. gonorrhoea		16	<0.12	<0.12
B. subtilis		16	<0.12	<0.12
Pr. mirabilis M92	11	>128	4	2
Ps. aeruginosa 8295	26	>128	>128	>128
Str. pyogenes		>128	<0.12	<0.12

Table 2. Resistance to alafosfalin among clinical isolates expressed as the percentage of strains with an MIC of >32 µg/ml

Organism		Total no. of strains	% Of strains with an MIC of >32 µg/ml with the following antibiotics:						
			Al	Am	Cx	Me	Tc	Na	Co
Gram-negative	*E. coli*	81	0	27	1	0	12	3	3
	K. aerogenes	41	2	41	0	5	20	10	3
	Enterobacter sp.	41	5	34	34	10	10	5	7
	S. marcescens	14	0	50	64	0	43	0	21
	S. typhimurium	26	0	0	0	0	0	0	0
Gram-positive	*S. aureus*	39	10	0	0	56	3	54	3
	S. albus	37	3	0	0	16	32	14	11
	Micrococcus sp.	36	20	0	0	6	6	100	0
	S. faecalis	41	0	20	100	100	13	100	0

Abbreviations: Al, alafosfalin; Me, mecillinam; Am, ampicillin; Cx, cephalexin; Tc, tetracycline; Co, cotrimoxazole; Na, Nalidixic acid.

organism was cultivated so that populations with resistance to alafosfalin, cephalexin or a combination of these two antibacterials could be selected. Resistance developed to these agents but it was greatly reduced, particularly for alafosfalin, by combination with cephalexin (ATHERTON et al., 1981) or mecillinam (GREENWOOD and VINCENT, 1979). The influence of the emergence of resistance on the duration of effect of alafosfalin on *E. coli* and on *Proteus mirabilis* has been measured in a mechanical model of the infected urinary bladder (GREENWOOD and O'GRADY, 1978). The result was similar to that observed with nalidixic acid and mecillinam (O'GRADY, personal communication). The development of resistance to agents such as nalidixic acid (STAMEY and BRAGONIE, 1976), streptomycin (TSENG et al., 1972) and mecillinam (GREENWOOD and O'GRADY, 1973) in vitro, has not prevented the effective use of these agents, clinically.

Synergy. It has been shown that there is synergy between alafosfalin and other agents, such as D-cycloserine and β-lactams, which have an effect on cell-wall biosynthesis. Over 500 clinical isolates from a wide range of genera have been studied (ALLEN et al., 1979b), using the well-known FICI-isobologram technique. Some results are summarized in Table 3.

The extent of synergy varied greatly with genus and with antibacterial agent. The combination alafosfalin–mecillinam showed good to excellent synergy against more than 80% of *Staphylococcus aureus* strains but less ($<30\%$, FICI <0.5) against intrinsically susceptible Gram-negative organisms. On the other hand, there was little synergy for *S. aureus* with the combination alafosfalin–ampicillin. Further synergy data using Japanese clinical isolates have been reported by MARUYAMA et al. (1979). It is interesting that very small amounts of alafosfalin convert cephalexin from a bacteriostatic into a bactericidal agent (ATHERTON et al., 1981).

Table 3. In vitro synergy between alafosfalin and three β-lactam antibiotics

Organism	β-lactam antibiotic	MIC with β-lactam antibiotic μg/ml	MIC with alafosfalin μg/ml	MIC with combination μg/ml	FICI	% Of all strains with FICI <0.5
E. coli S44	Mecillinam	0.03	0.03	0.0035 + 0.0035	0.23	31
Enterobacter OG3		0.12	0.5	0.024 + 0.096	0.39	16
K. aerogenes KA6		2	1	0.33 + 0.17	0.33	25
S. marcescens SM7		16	16	0.5 + 0.5	0.062	21
S. aureus R1		128	32	13 + 3.2	0.2	83
E. coli EC50	Cephalexin	8	0.25	1.9 + 0.061	0.48	19
Enterobacter OG3		16	1	1.88 + 0.12	0.24	27
K. aerogenes R16		16	2	1.8 + 0.22	0.22	25
S. aureus SAR6		4	64	0.47 + 7.5	0.23	44
Micrococcus MG3		8	64	1.77 + 14.2	0.42	36
E. coli S114	Ampicillin	1	0.06	0.24 + 0.015	0.48	21
Enterobacter OG4		8	4	0.67 + 0.33	0.17	33
K. aerogenes R2		16	8	2.66 + 1.33	0.33	15
S. aureus SA22		1	32	0.24 + 7.76	0.48	15
Micrococcus T14		0.5	16	0.06 + 1.9	0.24	47

Spheroplasts. In the presence of alafosfalin (100, 500 μg/ml) *Proteus mirabilis* cultures showed complete conversion to large spheroplasts after 8 h. These spheroplasts were viable and reverted to rods in alafosfalin-free broth. At lower concentrations the typical penicillin "bow-tie" forms and filaments were observed together with lozenge-shaped forms typical of mecillinam action. Spheroplasts were formed with other Gram-negative rods.

Turbidimetric studies with susceptible Gram-negative strains showed that alafosfalin was bactericidal at 4–8 times the M.I.C., but for Gram-positive organisms it was generally bacteristatic.

Experimental Studies in Animals

The activity of alafosfalin in vivo has been demonstrated (ALLEN et al., 1979b) against *E. coli, K. aerogenes,* and *S. fecalis,* using the mouse septicema model (Table 4). Unpublished investigations have confirmed activity, also, in a mouse model using *Enterobacter cloacae, Salmonella sp., Citrobacter sp.,* and *S. aureus* (CLEELAND, personal communication). The potency, in vivo, reflects the order of antibacterial activity observed in the in vitro studies.

Pharmacokinetic investigations in mice dosed with alafosfalin subcutaneously (ALLEN et al., 1979a) indicated rapid absorption and rapid elimination (half-life 10–11 min). For rats (sc) the half-life was approximately 20 min, for baboons 1 h. Alafosfalin was well absorbed, orally, but there was substantial first-pass metabolism in these animals. Peroral administration of the drug at doses up

Table 4. In vivo activities of alafosfalin and selected β-lactam antibiotics against infections in the mouse septicemia model

Organism	Subcutaneous CD_{50} (mg/kg)[a]			
	Alafosfalin	Ampicillin	Mecillinam	Cephalexin
E. coli 1346	6.2 (5.0–7.5)	0.8 (0.6–1.0)	2.8 (1.2–6.4)	9.2 (7.0–12.2)
E. coli 257	9.7 (7.8–12.1)	ND[b]	ND	ND
E. coli 5152[c]	52.3	>500	>250	ND
K. aerogenes 2	37 (28–49)	162 (123–214)	>400	25 (18–33)
K. aerogenes 9	35 (25–50)	>400	>400	>400
K. pneumoniae 503–988[c]	66.5	12	6	ND
E. cloacae 9456[c]	79	196	27	ND
P. mirabilis 190[c]	939	5	17	ND
P. vulgaris 48[c]	57	ND	0.71	ND
C. freundii 8ASM[c]	176	>500	>500	ND
S. faecalis 404	38 (31–48)	6.5 (3.8–11.1)	ND	>400
S. aureus (Smith)[c]	25	ND	ND	ND
S. aureus (Schoch)	>400	1.3 (0.7–2.3)	>400	26 (14–49)

[a] CD_{50}, 50% curative dose. Numbers in parentheses are 95% confidence limits.
[b] ND, Not determined.
[c] Data were obtained by R. CLEELAND et al. with the mouse septicemia model

to 1 g/kg indicated that the plasma concentration was directly related to the dose. The analysis of rat tissue 1 h after administration established that high levels of [^{14}C]-alafosfalin were in the kidney, plasma, lung and small intestine but relatively low concentrations were in the liver and central nervous system. A significant proportion of the dose was excreted as unchanged drug for all the species investigated. Alafosfalin, po, may be absorbed from the intestine by the action of a facilitated transport system rather than by diffusion. This is suggested by the more efficient absorption of the phosphonodipeptide than L-Ala(P), and evidence that the uptake process could be saturated. Moreover, experiments using alafosfalin in the presence of small natural peptides indicate competition for this transport system (ALLEN, unpublished).

Studies in Man

Pharmacokinetics. When the pharmacokinetic studies in the mouse, rat, and baboon were extended to man (ALLEN and LEES, 1980), somewhat similar results were obtained. The drug was well absorbed from the gastro-intestinal tract and approximately half of a 500 mg dose reached the general circulation, the remainder was hydrolyzed to L-alanine and L-aminoethylphosphonic acid. It was found that first-pass metabolism was less than in the case of the animal studies. Unchanged drug and metabolite are mainly excreted. The concentration

of alafosfalin excreted in the urine of healthy volunteers was dose dependant; recovery from urine for doses of 50 mg and 2500 mg increased from $6 \pm 1\%$ to $17 \pm 1\%$. In the case of subjects with impaired glomerular function less alafosfalin was excreted.

The rates of absorption and elimination of alafosfalin have been compared with those of cephalexin to provide a basis for use of combinations. It has been shown in in vitro studies that such combinations reduced the potential for development of resistant strains. When these two antibacterials were co-administered to healthy volunteers, both compounds were absorbed, distributed, and eliminated at closely similar rates. Oral administration of 500 mg alafosfalin with 250 mg cephalexin gave approximately equal concentrations of the drugs in plasma and a fourfold excess of cephalexin in the urine. Such a combination is envisaged for the therapy of urinary tract infections.

Clinical Studies

Clinical trials of alafosfalin administered by im and oral routes have been carried out in several centers in Europe and South America. In all studies, alafosfalin was exceptionally well tolerated. There was a very low incidence of side effects.

In the management of acute enteropathic *E. coli* diarrhoeal disease of infants (HIDALGO, unpublished) intramuscular doses of 10 mg/kg/day resulted in an overall success rate similar to conventional gentamicin therapy; alafosfalin treatment was successful in approximately 80% of cases. Further randomized studies comparing alafosfalin with bactrim and gentamicin (HIDALGO) and with chloramphenicol (BWIBO, unpublished) indicated that the drug was at least as effective as standard agents for the treatment of acute bacterial enterocolitis in infants, children, and adults, and for the treatment of shigellosis in children and adults. (ORTEGA and RAMIREZ; MEYRAN, unpublished results).

Mechanism of Action of Alafosfalin

Investigations by STROMINGER and others had established by the middle 1960's that penicillins and related β-lactams owed their antibacterial activity to inhibition of bacterial cell-wall biosynthesis (TIPPER and STROMINGER, 1965; OSBORN, 1969). At the molecular level this was attributed to a similarity of the molecular conformation of the penicillin to the D Ala D Ala fragment involved in cross-linking, an essential process in the formation of the bacterial cell wall. Penicillin inhibited the bacterial transaminase which accepted as a substrate the terminal $-$D Ala D Ala segment regularly occurring in the pentapeptide precursor of the cross-linked cell-wall structure (Fig. 1).

These studies on the course of biosynthesis of the bacterial cell wall and evidence that effective antibiotics such as D-cycloserine and vancomycin, as

Table 5. Effect of L- and D-Ala(P) on cell-wall enzymes[a]

Enzyme	K_m (mM)	K_i (mM)			Ratio of K_m to K_i		
		L-Ala(P)	D-Ala(P)	D-Cycloserine	L-Ala(P)	D-Ala(P)	D-Cycloserine
Alanine racemase (*E. coli*)	0.93	0.03	0.01	0.65	30	90	1.4
Alanine racemase (*S. faecalis*)	9	Irrev. (0.4)[b]	Irrev. (0.2)	Irrev. (0.1)			
UDP-NAMA-L-Ala synthetase (*S. aureus*)	0.3	3	Inact.[c]	Inact.	0.1		
D-Ala-D-Ala synthetase (*S. faecalis*)	0.5 20	Inact.	0.6 3	0.05 0.1		1 7	10 200
D-Ala-D-Ala adding enzyme (*S. faecalis*)	0.1	Inact.	Inact.	Inact.			

[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.
[b] Irrev., Irreversible inhibition. Rate constants for inactivation were 4.5×10^{-2} min^{-1} at 1 mM L-Ala(P) and 20×10^{-2} min^{-1} at 5 mM L-Ala(P). Apparent K_i values are shown in parentheses.
[c] Inact., Inactive at 10 mM

well as β-lactams, owed their effect to interference of biosynthesis arising from the action on the utilization of free or combined alanine, encouraged the idea that other compounds might be designed to act as antibacterials in this way. The Roche group in Britain selected the alanine-mimetic L-l-aminoethyl phosphonic acid from a range of alternatives they had investigated, and established that, combined with L-alanine to give the phosphonopeptide, alafosfalin, it inhibited the biosynthesis of the cell walls of various bacteria. In the earliest experiments this was indicated not only by the antibacterial activity of alafosfalin but also by its effect in producing spheroplasts and other aberrant forms.

The mechanism of action of alafosfalin has been elucidated (ATHERTON et al., 1979a). It has been established by experiments involving competition with natural di- and oligopeptides, as well as studies using isolated permease enzymes, that the first stage in the action of alafosfalin involves facilitated transport into the bacterial cell. The phosphonopeptide utilizes normal peptide transport mechanisms for L L-dipeptides (PAYNE, 1976).

The kinetic analysis of uptake of alafosfalin by the permease enzymes was investigated for *E. coli*. The non-linear, double reciprocal plot which was obtained indicated involvement of two non-interacting, saturable permeases. Similar data for Ala_2 and Ala_3 indicated involvement of two permeases in these cases. On the other hand, the double reciprocal plot for Ala Ala Ala (P) transport was consistent with the use of a single permease. It was shown that conversion of the natural peptide into phosphonopeptide caused at least tenfold decrease in affinity (ATHERTON et al., 1979a).

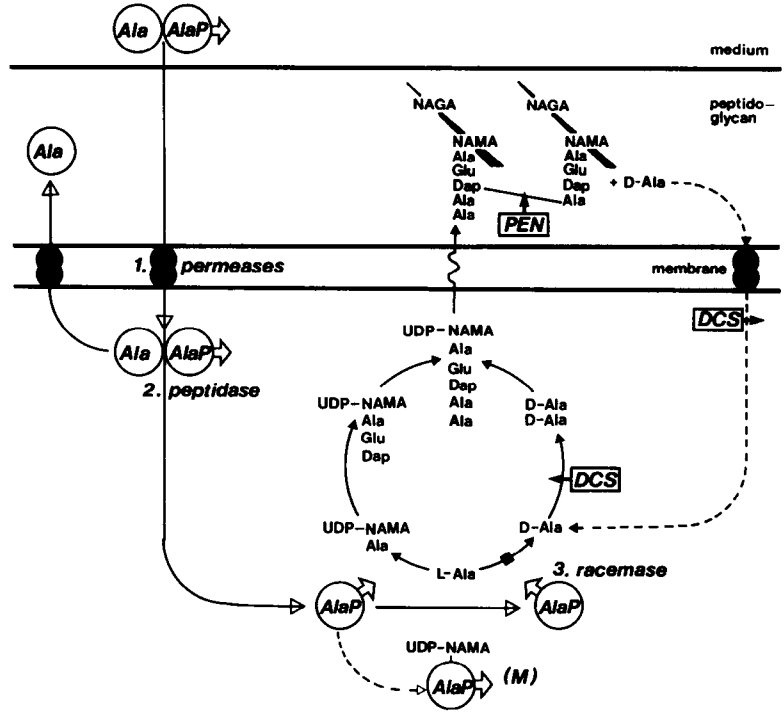


Fig. 1. Diagrammatic scheme for the mechanism of action of alafosfalin. *Abbreviations: Ala, AlaP,* alafosfalin; *DCS,* D-cycloserine; *PEN,* penicillin; *NAGA,* Nacetylglucosamine; *M,* metabolite M of Ala(P)

Differences in sensitivity and development of resistance of bacteria can be attributed largely to this first stage in the mechanism of action. As a consequence of this action of peptide permeases, alafosfalin is accumulated in the cytosol of the bacterium; the intracellular concentration may reach more than 1000 times that of the alafosfalin in the medium.

In the second stage the alafosfalin is converted, intracellularly by peptidases to L-1-amino-ethylphosphonic acid (L-Ala(P)) and L-alanine. [^{14}C]-labelling studies indicated that the L-Ala residue was not accumulated as for L-Ala(P) in the presence of chloramphenicol, presumably as a result of the preferential reversible action of the active transport on the natural amino acid. It was shown that L-Ala(P) was not transported into the cell to a significant degree. It has been established that the Ala(P) which is released acts at two points in the pathway of cell-wall peptidoglycan biosynthesis. It inhibits alanine racemase (LAMBERT and NEUHAUS, 1972) which is required for the conversion of L- to D-alanine, which is incorporated in the *terminal* residues -D Ala D Ala of the muramyl pentapeptide (Table 5).

Secondly, it interacts with UDP-NAMA in the presence of ligase (ITO et al., 1966), simulating L-alanine; further muramyl peptide chain extension is precluded since the phosphonic, unlike the carboxylic, function cannot form a