STUDIES ON THE SYNTHESIS OF CHEMOTHERAPEUTICS. PART XIII.[1] SYNTHESIS AND BIOLOGICAL STUDIES ON PHOSPHONOPEPTIDES HAVING ALKYL-, PHENYL-, AND HETEROCYCLIC SUBSTITUENTS.[2]

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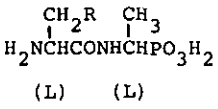
Abstracts —— New antibacterial phosphonopeptides (3) were synthesized in order to improve the stability against hydrolysis by peptidase and the antibacterial spectra. Synthesis of them was accomplished by condensation of the N-carbobenzoxyamino acid with diethyl aminoalkylphosphonate followed by deprotection and hydrolysis. The antibacterial activity was evaluated in a defined medium and the stability against hydrolysis by rat liver homogenates was examined. Chemical modification of the N-terminal amino acid moiety of the phosphonopeptide containing sulfur or oxygen atom at β- or γ-position of the N-terminal α-amino acid residue lost rapidly their in vivo activity in spite of the high in vitro activity. The para substituted phenylalanyl-1-aminoethylphosphonic acids showed higher stability and slightly lower activity compared with those of the corresponding ortho- and meta-substituted isomers. Some of these para-substituted compounds (e.g. 9β, 10β, and 19β) exhibited the same level of the biological activity as that of Alafosfalin (1) in potency of the activity,

broadness of the spectrum, and the serum level in mice after peroral administration. Phosphonopeptides consisted of unnatural amino acids and aminomethylphosphonic acid showed generally the extended spectra and activities, but these compounds were very fragile to hydrolysis by peptidase. None of phosphonopeptides containing 2-aminoethylphosphonic acid had the activity.

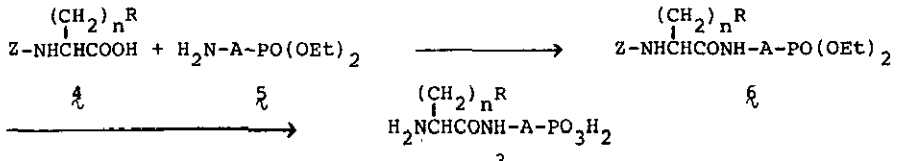
A synthetic phosphonopeptides, Alafosfalin (1, L-alanyl-L-1-aminoethylphosphonic acid) was introduced as a peptide mimetic which interferes with bacterisl cell wall synthesis with potent antibacterial activity especially against Gram negative bacilli.[3a-c] A good synergistic activity of 1 with β-lactam antibiotic was also found.[3d-i] In our preliminary stude,[4] approx. a hundred phosphonodi- and tripeptides containing mainly unnatural amino acid were synthesized. The antibacterial activities of some of these racemic phosphonopeptides were high and similar to that of Alafosfalin (1). However, further investigations have proved that our compounds and 1 in the previous papers[3a,4] suffered from hydrolysis by peptidases in serum of small intestine. This enzymic breakdown would cause low bioavailability of the phosphonopeptides.[3b,h-i] Alafosfalin (1) was selected as a promising synergistic chemotherapeutic by Roche's group on account of stability to hydrolysis by peptidease, while the L-methionyl analog (2) was abandoned because of fragility to the peptidase, although this compound (2) had the most potent in vitro antibacterial activity among the phosphonopepides containing natural amino acid. [3b]



(L) (L)

1 R = H
2 R = $-CH_2SCH_3$

The above findings stimulated us to intend further development of 1 and related compounds.[3,4] In this paper, new phosphonopeptides of the general formula 3 mainly consisting of unnatural amino acid were synthesized.



R = (substituted)phenyl, benzyl, heterocyclic alkoxy, or alkylthio group
A = $-CH(CH_3)-$, $-CH_2-$, or $-CH_2CH_2-$
n = 1 or 2
Z = carbobenzoxy

Scheme 1

Chemistry Synthetic method for phosphonopeptides (3) was essentially the same as that described in the previous paper[4] and represented by Scheme 1. Condensation of the N-carbobenzoxyamino acid (4) with the diethyl aminoalkylphosphonate (5)[5] in the presence of dicyclohexylcarbodiimide (DCC) gave the dipeptide (6). Deprotection of 6, followed by a hydrolysis with hydrogen bromide-acetic acid in the absence (Method A) or presence of a cation scavenger (Method B) afforded the objective phosphonopeptide (3) listed in Table 1 - 4. Unnatural amino acids for the left-half moiety were alternatively prepared as described in Experimental part.

Antibacterial Activity In vitro antibacterial activities of the synthesized phosphonopeptides were examined in a defined medium[3g] and compiled in Table 1 - 4 with their bacteriostatic index (B.I.).[6] Table 5 showed minimun inhibitory concentration (MIC) of the selected phosphonopeptides and known antibiotics.

Phosphonopeptides (8 - 21) consisted of the para-substituted phenylalanine and 1-aminoethylphosphonic acid (abbreviated to AEPA in this paper) showed the reduced activities of various degrees compared with that of the non-substituted compound (7). In the case of chlorine, bromine, and methylthio as the substituents, however, the activities of 9β, 10β, and 19β were almost equal to that of 7 (Table 5). It seemed that the activity was much reduced when the para-substituent became bulky or hydrophilic (e.g. 14, 15, 17, 20, and 21).

Compounds containing the alkoxy and alkylthio amino acid and AEPA (22 - 32) possessed generally the potent activities of average B.I. ≒ 4 (Table 2), and the spectra of 24, 26, and 28 were extended to Gram positive cocci (Table 5).

Phosphonopeptides (33 - 51) composed of amino acids and 1-aminomethylphosphonic acid (abbreviated to AMPA in this paper) preserved the high activities similar to the corresponding AEPA analogs (7 - 32) against Gram negative bacilli except for Pseudomonas aeruginosa (Table 3 and 5). The extended spectrum to Gram positive bacteria was characteristic for these AMPA series of compounds. Introduction of unnatural amino acid into the N-terminus was a good modification for increase of the activity. Antibacterial potency of phosphonopeptides consisted of natural amino acid was in the sequence of the Met, Phe, and Ala derivatives: This observation was true in both of the AEPA and AMPA series of compound.[3a] Para-substitution on the phenyl ring as seen in compounds 37 - 42 reduced somewhat the activity compared with the non-substitution in 35.

Phosphonopeptides (52 - 55) consisted of 2-aminoethylphosphonic acid which might be regarded as a β-alanine mimetic[7] did not show any of detectable activity (Table 4).

Stability against Enzymic Hydrolysis The stability against enzymic hydrolysis has been an important factor for peptide antibiotics to maintain their in vivo activity. The residual activities of the phosphonopeptides after treatment with rat liver homogenates (S-9) were examined (Table 6). Racemization of Alafosfalin as seen in compounds 56 and 57 did not influence on the stablity. Phosphonopeptides containing thiophene and furan rings (58 and 59) lost rapidly their activities by S-9 treatment, while Alafosfolin (1) retained the activity and its residual activity was estimated to be more than 50 % of the initial activity after 120 min. Methionyl compound 26, a racemized form of 2, lost its activity in a short time, and compound 7 and 60 were also labile to S-9.

In the case of the para-substituted phenylalanyl-AEPAs (9β - 19β), the stability was enhanced compared with the non-substituted compound (7). Especially, the nitro and carbamoyl compounds (16β and 18β) were more resistant against S-9 than 1. On the other hand, the AMPA series of the compounds (33 - 38, 41 and 44) was generally unstable to S-9 and lost rapidly their activities. The para-substitution on the phenyl-ring as seen in compounds 37, 38, and 41 did not contribute to the stability in contrast to the case of the AEPA analogs.

Inactivation of the phosphonopeptides was further tested using purified enzymes (Table 7). There was found that carboxypeptidase A was not effective for the inactivation. Leucine aminopeptidase brought about remarkably the inactivation as observed in the above S-9 treatment, but the inactivation time was dependant on a concentration of the enzyme. Alafosfalin (1), 9β , and 16β deminished slowly their activities and compound 58 lost rapidly the activity by the S-9 and leucine aminopeptidase.

The structural isomerism of some arylalanyl-AEPAs was found to change the rate of the inactivation by S-9 as shown in Fig. 1. The para-fluoro compound (8) was more stable than the ortho- and meta-fluoro compounds (61 and 62) and the 4-pyridyl compound (65) was the most stable one among three isomeric pyridyl-AEPAs. However, their antibacterial potency was in inverse proportion to their stability: The stable isomers (8 and 65) showed the lowest activity in each of the phenyl- and pyridylalanyl-AEPAs, respectively.

Comparison of the inactivation rate between the phosphonopeptides and clinically used antibiotics was shown in Fig. 2. The activities of Cephalothin and Polymyxin-B were rapidly reduced but remained on the levels of 7 % (approx.) of the initial activity after 120 min. The activities of D-cycloserine and Cephalexin were not

affected by S-9, and Fosfomycin magnified of its activity. Alafosfalin (1), 10β, and 16β were gradually inactivated by 50 - 75 % of the initial activity at 120 min.

Serum Level of Phosphonopeptides in Mice In connection with the in vitro inactivation experiments above, serum levels of the phosphonopepiteds were examined in order to search for the pharmacokinetics. All phosphonopeptides examined were absorbed immediately after peroral administration to mice and the peak concentrations were found within 15 min of the dosing. However the different serum levels among the examined compounds were observed (Fig. 3). The serum level of Alafosfalin (1) declined exponintically, with half-life of approx. 20 min, while the compound 7 showed the parallel but lowered serum level compared with that of 1. Para-substituted phenylalanyl-AEPAs (9β, 10β, and 16β) which were resistant to the S-9 or leucine aminopeptidase above showed the highest serum level among the examined phosphonopeptides. Especially, the compound 16β maintained a high serum level with prolonged half-life of approx. 30 min. Unstable phosphonopeptides against the S-9 (35, 58, and 59) had undetectable serum concentration at the period of 120 min after peroral administration of doses up to 400 mg/kg to mice.

Discussion It is acknowledged that phosphonopeptides, represented by Alafosfalin (1), should pass two essential stages for exhibiting the in vitro antibacterial activity; (i) active transport from the medium into the bacterial cell and (ii) intracellular cleavage to release alanine mimetics, e.g. L-AEPA.[3c,g] If the cleavage to release AEPA occurred before transporting of the phosphonopeptide into the bacterial cell, AEPA itself could not be involved in active transport system which carries intact phosphonopeptide. Furthermore, phosphonopeptide transported into the bacterial cell would not show any antibacterial activity when they were not cleaved by intracellular bacterial peptidase.

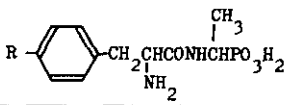
A structural variation in the N-terminus of the AEPA and AMPA series of phosphonopeptides influenced greatly on the antibacterial potency and spectra. Phosphonopeptides containing alkylthio, alkoxy, some para-substituted phenyl, or heterocyclic group on the N-terminal amino acid residue showed generally high activities with extended spectra to Gram positive bacteria in some cases. Retention of these high activities might be rationalized by an additional blockade of the bacterial enzymes owing to the left-half unnatural amino acid as an amino acid mimetic.[8]

A structural difference of the phosphonic acid moiety between the AEPA and AMPA series of phosphonopeptides was responsible for the antibacterial spectrum. The AEPA series of compounds inhibited generally the growth of Gram negative bacteria except for that

of Pseudomonas aeruginosa and showed few activity against the Gram positive bacteria. The antibacterial spectrum of the AMPA series of compounds was broadened to the Gram positive bacteria. This spectral difference between the AEPA and AMPA series of phosphonopeptides would be attributed to a reported observation that L-alanyl-AMPA (33) was transported into the bacterial cell more easily than Alafosfalin (1).[3a] Phosphonopeptide as the chemotherapeutic agent or synergist should be resistant against peptidase outside the bacterial cell and possess the good pharmacokinetics.[3d-f] It would be essential for the evaluation of chemotherapeutic phosphonopeptides to examine their stability to the peptidases. In this paper, rat liver homogenate (S-9) was shown to be useful to examine the stability without using purified enzyme. This simple method provided the following results: (a) The phosphonopeptide was enzymatically hydrolysed to give α-amino acid and AEPA (or AMPA),[9] thus inactivated. (b) This inactivation by the hydrolysis was more or less inevitable for the examined phosphonopeptides. (c) The AEPA series of compounds were generally more resistant against the hydrolysis than those of the AMPA series. The stability of para-substituted phenylalanyl-AEPAs (9β, 10β, and 16β) was well reflected on their higher serum levels and peak concentrations than those of Alafosfalin (1). These compounds had the same level of the antibacterial activities as that of 1.

The above results and discussion have shown a structure-activity relationship of the phosphonopeptides including the relations between the antibacterial activity and the inactivation by peptidases. Further investigation on the synergistic activity with aminoglycosides and β-lactam antibiotics and the in vivo activity are in progress.

Table 1: Phosphonopeptides Having Para-Substituted Phenylalanine Moiety



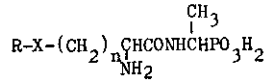
Compd. No.	R[a)]	Method	Yield (%)	mp (dec.) °C	Average B. I. Gram(-)	Gram(+)	Total
7	H (L)	A[b)]	92.0	278-280	4.6	1.3	3.9
8	F	A[b)]	84.0	250-253	2.9	0.7	2.4
9β[c)]	Cl	A	83.8	231-233	4.6	0.7	3.8
10α	Br	A	79.5	258-259	0.6	0	0.5
10β	Br	A	85.7	235-236	4.5	0.7	3.7
11α	I	A	88.7	262-263	0.8	0	0.7
11β	I	A	73.0	239-241	3.6	0.3	2.9
12	OH	A	87.5	265-290	1.6	0	1.3
13	CH_3	A[b)]	82.0	249-252	2.8	0	2.2
14	CH_3O	A	91.8	242-245	1.4	0.7	1.3
15	C_2H_5O (L)	A	64.0	246-250	2.1	0	1.7
16α	NO_2	A	73.9	261-262	0.8	0	0.6
16β	NO_2	A	94.0	256-258	3.4	0.3	2.8
17	NH_2	A[b)]	82.0	287-292	2.3	0	1.8
18α	$NH_2C(=O)-$	A	89.3	289-290	0.8	0	0.6
18β	$NH_2C(=O)-$	A	77.3	262	3.1	0	2.5
19α	CH_3S	A	76.4	255-257	2.8	0	2.2
19β	CH_3S	A	58.6	235-238	4.6	0.7	3.8
20α	C_2H_5S	A	52.8	257-259	0.1	0	0.1
20β	C_2H_5S	A	51.4	234-236	2.0	0.7	1.7
21	$(CH_3)_2N-$	A[b)]	92.0	244-247	0.7	0	0.5

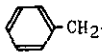
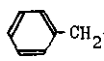
a) Some of N-terminal amino acids used as starting materials are L-form as indicated in the parentheses, and the others unspecified are recemic, thus giving the diastereoisomeric mixture of racemic form.

b) Synthesis of this compound was also reported in the previous paper[4)].

c) Synthesis of the α-isomer was not examined.

Table 2: Phosphonopeptides Having Alkylthio and Alkoxy Substituents



Compd. No.	R[a)]	X	n	Method	Yield (%)	mp (dec.) °C	Average B. I. Gram(-)	Gram(+)	Total
22		S	1	B	58.5	237-241	4.8	0.7	4.0
23	 (L)	S	1	B	73.0	236-239	5.4	2.0	4.7
24[b)]	CH_3- (L)	S	1	A	65.6	223-235	6.5	2.0	5.6
25		S	2	B	88.5	235-253	2.8	0.3	2.3
26	CH_3- (L)	S	2	B	67.0	225-235	6.1	4.3	5.7
27α	CH_3CH_2-	O	2	A	quant.	233-240	1.3	0	1.1
27β	CH_3CH_2-	O	2	A	89.0	171-178[c)]	5.3	0.7	4.4
28		O	2	A	94.0	235-239	5.3	3.0	4.9
29	CH_3-	O	1	A	64.3	190-195	3.1	0	2.5
30	Br- 	O	2	A	43.5	228-231	4.1	1.7	3.6
31	CH_3-	S=O	2	A	37.0	165-180	1.6	0	1.3
32	CH_3-	O=S=O	2	A	49.3	201-220	0.4	0	0.3

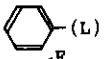
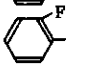
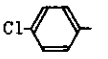
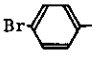
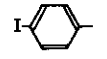
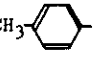
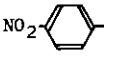
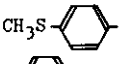
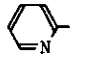
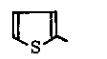
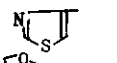
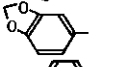
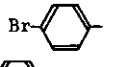
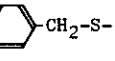
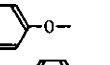
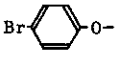
a) See footnote a) in Table 1.

b) See footnote b) in Table 1.

c) Melted without decomposition

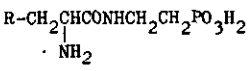
Table 3: Phosphonopeptides Having Aminomethylphosphonic Acid Moiety

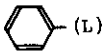
$$R\text{-}(CH_2)_n\underset{NH_2}{\overset{|}{C}}HCONHCH_2PO_3H_2$$

Compd. No.	R[a)]	n	Method	Yield (%)	mp (dec.) °C	Average B. I. Gram(-)	Gram(+)	Total
33	H (L)	1	A	78.0	180-188	2.3	2.7	2.4
34	CH_3	1	A	99.0	185-190	3.4	3.3	3.4
35	 (L)	1	A	98.0	246-250	3.9	3.7	3.9
36		1	A	88.0	262-264	3.3	2.0	3.0
37		1	A	73.3	260-262	3.6	1.7	3.2
38		1	A	69.1	247-248	3.5	1.7	3.1
39		1	A	75.7	240-242	2.7	1.3	2.4
40		1	A	72.2	255-259	2.1	1.7	2.0
41		1	A	77.9	264-266	2.3	1.3	2.1
42		1	A	64.4	242-243	3.3	1.0	2.9
43		1	A	57.9	180-185	3.2	3.7	3.3
44		1	A	87.5	256-259	3.9	3.3	3.4
45		1	A	59.0	168-170	3.4	5.0	3.7
46		1	A	quant.	215-217	2.7	1.3	2.4
47		2	A	80.0	272-276	2.8	1.3	2.5
48		1	B	77.8	243-247	4.6	3.3	4.3
49	CH_3-S- (L)	2	B	43.5	243-247	5.0	5.3	5.1
50		2	A	86.0	254-256	4.5	4.0	4.4
51		2	A	89.0	252-255	3.3	3.0	3.3

a) See footnote a) in Table 1.

Table 4: Phosphonopeptides Having 2-Aminoethylphosphonic Acid Moiety[a)]



Compd. No.	R	Method	Yield (%)	mp (dec.) °C	Average B. I. Gram(-)	Gram(+)	Total
52	H (L)	A	45.9	267-270	0	0	0
53	 (L)	A	57.0	278-280	0	0	0
54		A	36.0	274-278	0	0	0
55	CH_3O-	A	41.7	230-233	0	0	0

a) See footnote a) in Table 1.

Table 5: Antibacterial Activities of Phosphonopeptides (MIC, μg/ml)

	7	9β	10β	16β	24	26	28	35	45	49
Escherichia coli NIHJ	0.39	0.39	0.78	0.78	≤0.05	≤0.05	0.20	0.39	1.56	0.20
E. coli IAM 1182	0.39	0.78	0.78	0.78	≤0.05	≤0.05	0.39	3.13	6.25	0.39
Shigella flexneri 2a EW 10	3.13	0.78	0.78	6.25	0.39	0.39	0.78	6.25	3.13	1.56
Shi. sonnei EW 33	0.20	0.20	0.20	0.20	≤0.05	≤0.05	0.10	0.78	3.13	0.39
Salmonella typhosa 0901	≤0.05	≤0.05	≤0.05	0.20	≤0.05	≤0.05	≤0.05	0.39	0.39	0.10
Sal. enteritidis G 14	25	50	25	>100	12.5	12.5	12.5	50	100	12.5
Klebsiella pneumoniae Kasuya	6.25	3.13	3.13	12.5	0.39	1.56	3.13	6.25	12.5	3.13
Proteus vulgaris OX 19 US	>100	>100	>100	>100	25	50	>100	100	>100	25
Serratia marcescens IID 618	3.13	1.56	3.13	25	0.20	0.39	1.56	1.56	3.13	1.56
Ser. narcescens IID 619	6.25	12.5	25	>100	0.78	0.78	12.5	6.25	6.25	3.13
Pseudomonas aeruginosa P 1	>100	>100	>100	>100	50	50	>100	>100	>100	>100
Pse. aeruginosa P 31	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Staphylococcus aureus ATCC 6538P	>100	100	>100	>100	>100	6.25	100	25	6.25	3.13
S. aureus Terajima	50	25	50	50	12.5	3.13	25	3.13	3.13	1.56
Bacillus subtilis PCI 219	12.5	>100	>100	100	12.5	6.25	>100	6.25	1.56	3.13

Table 5: continued

	50	1 (Alafosfalin)	FOM[a)]	ABPC[b)]	CS[c)]
Escherichia coli NIHJ	0.20	0.78	100	0.39	25
E. coli IAM 1182	3.13	0.39	100	3.13	25
Shigella flexneri 2a EW 10	3.13	1.56	50	1.56	25
Shi. sonnei EW 33	0.78	0.10	12.5	3.13	12.5
Salmonella typhosa 0901	≤0.05	0.10	1.56	0.39	3.13
Sal. enteritidis G 14	25	25	25	0.78	50
Klebsiella pneumoniae Kasuya	6.25	3.13	50	12.5	12.5
Proteus vulgaris OX 19 US	>100	>100	50	50	100
Serratia marcescens IID 618	0.78	3.13	12.5	25	>100
Ser. marcescens IID 619	3.13	3.13	100	100	>100
Pseudomonas aeruginosa P 1	>100	>100	>100	>100	100
Pse. aeruginosa P 31	>100	>100	25	50	100
Staphylococcus aureus ATCC 6538P	3.13	>100	25	0.10	6.25
S. aureus Terajima	3.13	25	25	0.20	3.13
Bacillus subtilis PCI 219	25	>100	>100	≤0.05	3.13

a) Fosfomycin, b) Ampicillin, c) D-Cycloserin

Table 6: Stability of Phosphonopeptides against Enzymic Hydrolysis by Rat Liver Homogenates (S-9)

Compd. No.[a]	Residual Activity (%)[b]			
	15min	30min	60min	120min
1	94.0	90.0	72.0	52.0
56	c)	c)	68.0	55.0
57	c)	c)	76.0	52.5
7	17.0	1.5	0.2	0
9β	c)	62.0	41.5	25.0
10β	85.0	77.0	55.0	36.5
11β	76.0	66.0	48.0	28.5
12	c)	49.5	26.0	5.9
13	c)	50.0	27.5	10.0
14	c)	65.0	55.0	42.5
16β	88.0	74.0	70.0	63.0
17	c)	37.0	25.5	8.3
18β	103.0	94.0	80.0	62.0
19β	c)	60.0	47.0	24.0
24	0	0	0	0
26	2.6	0.2	0	0
58	1.1	0	0	0
59	0	0	0	0
60	19.3	2.3	0	0
33	40.0	14.3	0	0
34	18.2	2.7	0	0
35	0	0	0	0
36	0	0	0	0
37	0	0	0	0
38	0	0	0	0
41	0	0	0	0
44	0	0	0	0

a) 56, DL-alanyl-DL-AEPA; 57, L-alanyl-DL-AEPA; 58, β-2-thienyl-DL-AEPA; 59, β-2-furanyl-DL-AEPA; 60, α-aminobutyryl-DL-AEPA. Synthesis of these compounds was previously reported[4].

b) Residual activity of the test compound was expressed as the percentage of residual active concentration after enzymic hydrolysis to initial concentration (100 μg/ml). Zero percent showed no detection of the residual activity.

c) Not determined.

Table 7: Inactivation of Phosphonopeptides by Peptidase

Compd. No.[a]	Leucine Aminopeptidase			Carboxypeptidase A		
	Concn. of Peptidase (μg/ml)	Residual Activity (%)[b]		Concn. of Peptidase (μg/ml)	Residual Activity (%)	
		60min	120min		60min	120min
1	125	69	47	500	93	93
	25	94	83	100	102	96
	5	102	78	20	98	94
7	25	23	7	c)	c)	c)
9β	25	84	61	c)	c)	c)
16β	25	94	88	c)	c)	c)
53	125	0	0	500	107	105
	25	0	0	100	109	112
	5	24	0	20	104	95

a) See footnote a) in Table 6.

b) See footnote b) in Table 6.

c) Not determined.

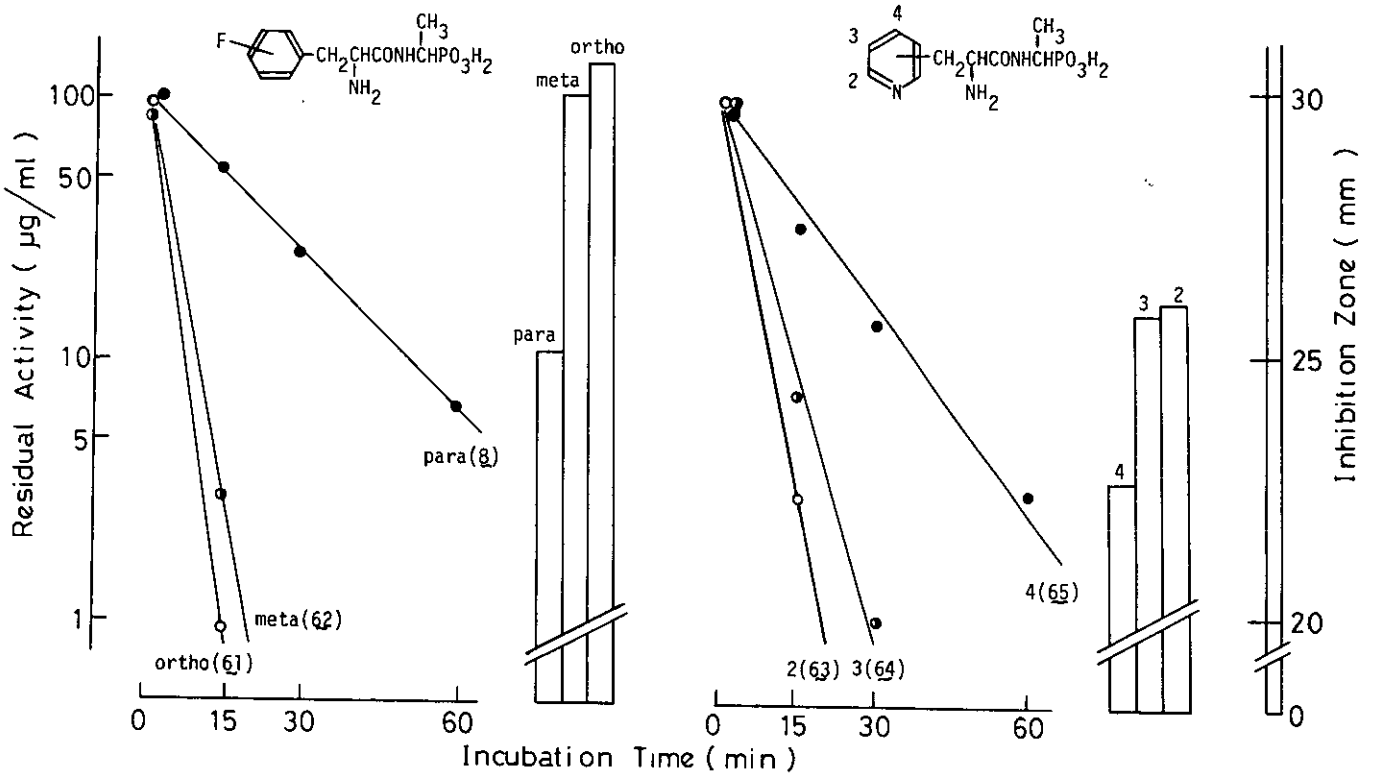


Fig. 1: Antibacterial Activity and Stability against the S-9 Hydrolysis of Isomeric Arylalanyl Phosphonopeptides. Activity was expressed by the diameter of inhibition zone at 100 μg/ml of the test compound against Shi. sonnei EW 33, and stability was expressed by the residual activity after treatment by the S-9.
61, o-fluorophenylalany-DL-AEPA; 62,m-fluorophenylalanyl-DL-AEPA; 63, 2-pyridyl-DL-AEPA; 64, 3-pyridyl-DL-AEPA; 65, 4-pyridyl-DL-AEPA. Synthesis of these compounds was previously reported.[4)]

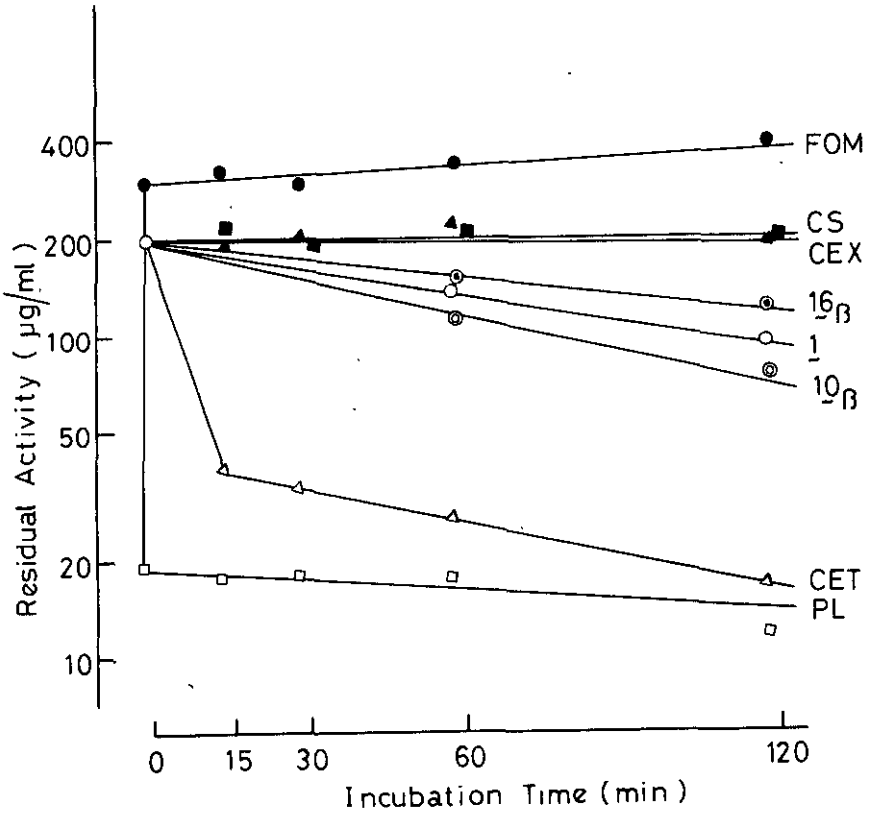


Fig. 2: Inactivation of Phosphonopeptides and Clinically Used Antibiotics by Rat Liver Homogenates(S-9). Initial concentration of the test compounds was 200 μg/ml. CEX, Cephalexin; CET, Cephalothin; FOM, Fosfomycin; CS, D-Cycloserine; PL, Polymyxin-B.

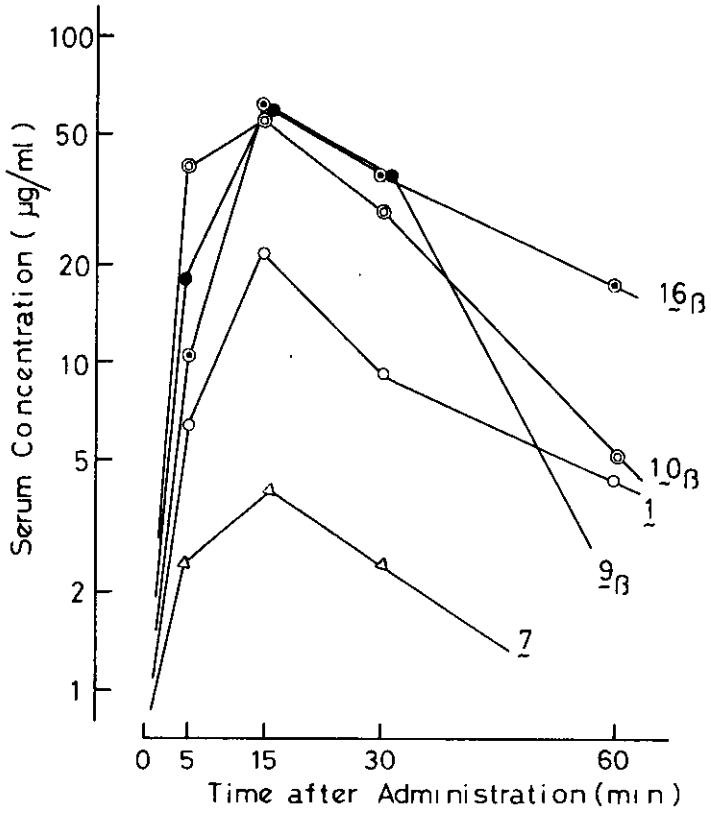


Fig. 3: Serum Levels of Phosphonopeptides after Peroral Administration to Mice (Dosage: 100 mg/Kg). Serum concentrations were not detected at lowered concentrations; 1 1.0, 7 2.0, 9β 2.0, 10β 2.0, and 16β 5.0 μg/ml.

EXPERIMENTAL

All melting points are not corrected. IR spectra were determined on a Hitachi 215 recording spectrometer and NMR spectra on a JNM PMX-60 spectrometer using tetramethylsilane or sodium trimethylsilylpropanesulfonate as an internal standard. Reference antibiotics (AB-PC, D-Cycloserine, CEX, CET, and Polymyxin-B) and authentic peptidases (leucine aminopeptidase and carboxypeptidase-A) used were Sigma reagents of Sigma Chemical Co., USA. Fosfomycin used was Fosmicin-S® for injection of Meiji Seika, Co., Japan. Alafosfalin was supplied from the Roche Products, UK.

Starting Amino Acids

a) The following amino acids were prepared according to the literature shown: β-4-iodo-D,L-phenylalanine,[10] β-4-nitro-D,L-phenylalanine,[11] β-4-methylthio-, and β-4-ethylthio-D,L-phenylalanine,[12] S-phenyl-D,L-cysteine,[13] S-benyl-D,L-homocysteine,[14] O-ethyl-D,L-homoserine,[15] O-phenyl-D,L-homoserine.[16]

b) The other amino acids were commercially available or prepared by the usual method for α-amino acid.[17] For example, condensation of 4-bromophenoxyethyl bromide with diethyl acetaminomalonate in the presence of sodium ethoxide followed by hydrolysis with a mixture of dil. HCl and AcOH gave O-4-bromophenyl-homoserine as colorless crystals, mp 228 - 231° (dec.).

N-Carbobenzoxy Amino Acids (4)

These compounds were prepared by usual procedure for carbobenzoxylation of α-amino acid; the free α-amino acid was treated with carbobenzoxy chloride in the presence of sodium hydroxide to obtain the N-protected amino acid after usual work-up.

Diethyl Aminoalkylphosphonates (5)

Diethyl D,L-1-aminoethylphosphonate was prepared by the following one pot procedure by modified Chalmer's method.[18] A mixture of acetaldehyde-ammonia 12.25 g (0.2 mol), diethyl phosphite 30.38 g (0.22 mol), and liquid ammonia 40 ml was stirred in a sealed tube at room temperature for 3 days. Distillation of the reaction product gave a colorless oil, 27.51 g (76.0 %), bp 58 - 61° (0.25 mmHg). Diethyl aminomethylphosphonate[19] and 2-aminoethylphosphonate[20] were respectively prepared by the reported method.

Diethyl N-Substituted Aminoalkylphosphonate Derivatives (6)

A mixture of equimolecular amounts of N-carbobenzoxy-α-amino acid, diethyl D,L-1-aminoalkylphosphonate (5), and DCC was stirred in an enough ammount of appropriate solvent (CH_2Cl_2, benzene, AcOEt, DMF or their mixture to dissolve the amino acid and DCC). The reaction temperature was maintained below 5° by ice-cooling for 0.5 - 3 hr

followed by coming up to room temperature, and the stirring was continued for a further 8 - 24 hr. A precipitated dicyclohexylurea was removed by filtration. The filtrate was washed (dil. HCl, water, and brine), dried (Na_2SO_4), and evaporated. The crude product, thus obtained, was subjected to fractional crystallization from appropriate solvents[21] or column chromatography[22] using silica gel to give pure product (optically active compound, racemate, diastereoisomeric mixture, or it's separated α- and β-isomer).

Phosphonopeptides (3) Method A

A mixture of the N-substituted aminoalkylphosphonate derivative (6) a g and excess 30 % HBr-AcOH 5 - 15 x a ml was stirred at room temperature for 12 - 64 hr and poured into ether. An oily substance precipitated was collected by decantation and taken up into EtOH or MeOH. To this solution was added an excess of propylene oxide to precipitate a solid which was collected by filtration and washed with MeOH to give an objective phosphonopeptide. This method provided the following phosphonopeptides as colorless crystals.

Compd. No.	IR (KBr) cm^{-1}	NMR (D_2O-NaOD) δ	
		$PCHCH_3$ or PCH_2-	Other protons
7	3270, 1650	0.96(dd, J=12.5, 7.0Hz), 1.18(dd, J=13.5, 7.0Hz)	7.35(5H, s, $-C_6H_5$)
8	3270, 1650	[a]1.32~1.77(m)	6.98~7.50(4H, m, F-C_6H_4-)
9β	3260, 1650	1.38(dd, 13.2, 7.0Hz)	7.25 and 7.44(each2H, d, J=8.4Hz, Cl-C_6H_4-)
10α	3250, 1650	1.16(dd, J=14.4, 7.2Hz)	7.21 and 7.54(each2H, d, J=8.0Hz, Br-C_6H_4-)
10β	3250, 1648	1.13(dd, J=13.9, 7.4Hz)	7.14 and 7.48(each2H, d, J=8.4Hz, Br-C_6H_4-)
11α	3260, 1654	1.12(dd, J=13.6, 7.2Hz)	6.97 and 7.62(each2H, d, J=8.0Hz, I-C_6H_4-)
11β	3240, 1654	1.35(dd, J=13.6, 7.0Hz)	6.97 and 7.62(each2H, d, J=8.0Hz, I-C_6H_4-)
12	3270, 1650	[a]1.43(dd, J=15.0, 7.0Hz)	7.01 and 7.30(each2H, d, J=8.5Hz, HO-C_6H_4-)
13	3270, 1650	1.04(dd, J=14.0, 7.5Hz), 1.24(dd, J=14.0, 7.5Hz)	2.37(3H, s, CH_3-C_6H_4-), 7.30(4H, s, CH_3-C_6H_4-)
14	3270, 1650	[a]1.23~1.81(m)	4.00(3H, s, $-OCH_3$), 7.01 and 7.27 (each2H, d, J=8.0Hz, CH_3O-C_6H_4-)
15	3280, 1650	0.99(dd, J=14.0, 7.5Hz), 1.22(dd, J=14.0, 7.5Hz)	1.39(3H, t, J=7.0Hz, $-OCH_2CH_3$), 4.07(2H, q, J=7.0Hz, $-OCH_2CH_3$), 6.97 and 7.27(each2H, d, J=9.0Hz, EtO-C_6H_4-)
16α	3260, 1652	1.02(dd, J=13.6, 7.2Hz)	No clear signals were observed.
16β	3250, 1647	[b]1.36(dd, J=16.0, 7.0Hz)	7.71 and 8.38(each2H, d, J=8.8Hz, NO_2-C_6H_4-)
17	3280, 1650	[a]1.17~1.56(m)	7.54(4H, s, H_2N-C_6H_4-)
18α	3270, 1650, 1610	0.91(dd, J=13.4, 6.8Hz)	7.38 and 7.82(each2H, d, J=8.0Hz, H_2NCO-C_6H_4-)
18β	3270, 1648, 1605	1.22(dd, J=13.4, 7.4Hz)	7.38 and 7.82(each2H, d, J=8.0Hz, H_2NCO-C_6H_4-)
19α	3250, 1648	1.22(dd, J=13.8, 7.6Hz)	2.44(3H, s, $-SCH_3$)
19β	3250, 1648	1.37(dd, J=13.8, 7.6Hz)	2.44(3H, s, $-SCH_3$)
20α	3270, 1652	1.17(dd, J=13.0, 7.0Hz)	1.25(3H, t, J=7.6Hz, $-SCH_2CH_3$), 2.89(2H, q, J=7.6Hz, $-SCH_2CH_3$)
20β	3250, 1652	1.36(dd, J=14.0, 7.4Hz)	1.25(3H, t, J=7.6Hz, $-SCH_2CH_3$), 2.89(2H, q, J=7.6Hz, $-SCH_2CH_3$)
21	3260, 1650	0.95(dd, J=14.0, 7.0Hz), 1.18(dd, J=14.0, 7.0Hz)	2.80[6H, s, $-N(CH_3)_2$], 6.96 and 7.23(each2H, d, J=9.0Hz, Me_2N-C_6H_4-)

24	3250, 1655	1.25(dd, J=13.8, 7.4Hz)	2.17(3H, s, $-SCH_3$)
27α	1690	1.23(dd, J=14.0, 7.0Hz)	1.17(3H, t, J=7.0Hz, $-OCH_2CH_3$)
27β	1660	1.26(dd, J=14.0, 7.0Hz)	1.20(3H, t, J=7.0Hz, $-OCH_2CH_3$)
28	3270, 1655	1.14(dd, J=14.0, 7.0Hz), 1.22(dd, J=14.0, 7.0Hz)	6.88~7.54(5H, m, $-C_6H_5$)
29	3270, 1650	[a]1.60(dd, J=17.5, 7.5Hz)	3.58(3H, s, $-OCH_3$)
30	3255, 1650	1.19(dd, J=13.5, 7.5Hz), 1.28(dd, J=13.5, 7.5Hz)	6.93 and 7.50(each2H, d, J=9.0Hz, $Br-C_6H_4-$)
31	1655	[c]1.34(dd, J=15.0, 7.0Hz)	2.80(3H, s, $-SOCH_3$)
32	3400, 1675	[c]1.33(dd, J=15.0, 7.0Hz)	3.16(3H, s, $-SO_2CH_3$)
33	1660	3.22(d, J=13.0Hz)	1.23(3H, d, J=7.0Hz, $-CHCH_3$)
34	1660	3.55(dd, J=12.0, 2.0Hz)	1.35(3H, t, J=7.0Hz, $-CH_2CH_3$)
35	1655	3.19(dd, J=12.0, 2.0Hz)	7.33(5H, s, $-C_6H_5$)
36	3260, 1655	3.22(dd, J=13.0, 4.0Hz)	7.08~7.55(4H, m, $F-C_6H_4-$)
37	3190, 1650	3.23(dd, J=13.0, 2.0Hz)	7.15~7.60(4H, m, $Cl-C_6H_4-$)
38	3250, 1655	2.90~3.75(m)	7.20 and 7.54(each2H, d, J=8.4Hz, $Br-C_6H_4-$)
39	3190, 1655	3.23(dd, J=13.0, 2.0Hz)	7.08 and 7.72(each2H, d, J=8.0, 2.0Hz, $I-C_6H_4-$)
40	3260, 1655	3.23(dd, J=13.0, 2.0Hz)	7.30(4H, s, $CH_3-C_6H_4-$)
41	3280, 1655	3.27(dd, J=13.0, 3.0Hz)	7.53 and 8.25(each2H, d, J=8.5Hz, $NO_2-C_6H_4-$)
42	3255, 1655	2.92~3.87(m)	2.43(3H, s, $-SCH_3$), 7.27(4H, s, $MeSC_6H_4-$)
43	3260, 1660	2.82~3.82(m)	7.32~7.66, 7.78~8.15 and 8.50~8.75 (each1H, m, pyridyl-H×3)
44	3270, 1660	3.26(d, J=12.5Hz)	6.96~7.24(2H, m, thienyl-H×2), 7.45 (1H, dd, J=5.0, 2.0Hz, thienyl-H)
45	3220, 1675	2.80~3.83(m)	7.50 and 9.07(each1H, d, J=2.0Hz, thiazolyl-H×2)
46	3255, 1655	3.19(dd, J=12.5, 2.0Hz)	6.03(2H, s, $-OCH_2O-$)
47	3255, 1660	3.26(dd, J=13.0, 2.0Hz)	7.18 and 7.52(each2H, d, J=8.0Hz, $Br-C_6H_4-$)
50	3250, 1650	3.26(dd, J=13.0, 2.0Hz)	6.95~7.66(5H, m, $-OC_6H_5$)
51	3250, 1655	3.28(dd, J=13.0, 2.0Hz)	6.97 and 7.56(each2H, d, J=9.0Hz, $Br-C_6H_4-$)
52	3300, 1655	[a]2.15~2.90(m)	1.76(3H, d, J=6.0Hz, $-CHCH_3$)
53	3260, 1655	[a]2.0~2.7(m)	7.43(5H, s, $-C_6H_5$)
54	3260, 1655	[a]2.0~2.7(m)	3.45(2H, d, J=7.0Hz, H_2NCHCH_2-)
55	3275, 1655	[c]1.46~2.17(m)	3.41(3H, s, $-OCH_3$)

a) NMR spectrum was determined in CF_3CO_2H, b) in CD_3CO_2D, c) in D_2O.

Method B: A mixture of the diethyl N-substituted aminoalkylphosphonate (6) 1 mol. eq., excess 30% HBr-ACOH, and methyl sulfide ca. 10 mol. eq. was stirred at room temperature for 20~24hr. The reaction mixture was worked up as in the case of Method A. This method provided the following phosphonopetides.

Compd. No.	IR (KBr) cm^{-1}	NMR (D_2O-NaOD) δ	
		$PCHCH_3$ or PCH_2-	Other protons
22	3255, 1650	1.22(dd, J=14.0, 7.5Hz)	7.30~7.75(5H, m, $-C_6H_5$)
23	3275, 1650	1.27(dd, J=13.0, 7.5Hz)	3.80(2H, s, $-CH_2C_6H_5$), 7.43(5H,s, $-C_6H_5$)
25	3260, 1650	1.23(dd, J=14.0, 7.0Hz)	3.81(2H, s, $-SCH_2C_6H_5$)
26	3270, 1650	a) 1.36(dd, J=15.0, 7.0Hz)	3.17(3H, s, $-SCH_3$)
48	3280, 1655	3.28(d, J=13.0Hz)	3.80(2H, s, $-SCH_2C_6H_5$), 7.48(5H, s, $-C_6H_5$)
49	1655	3.55(dd, J=13.0, 3.0Hz)	2.18(3H, s, $-SCH_3$)

a) NMR specturm was determined in D_2O.

Determination of Antibacterial Activity

The minimum inhibitory concentration (MIC) and bacteriostatic index (B.I.) caluculated by the equation: $\log_2[100/\text{MIC}\ (\gamma g/ml)]$[6] was determined on the defined agar by a method previously described.[4]

Enzymic Hydrolysis

A homogenate of rat liver was prepared as a crude peptidase by using a method similar to one described previously.[23] After homogenation of S.D. rat liver in 3 times volume of a cold KCl solution (0.15 M) followed by centrifugation of the homogenates at 9,000 x g for 10 min, the supernatant (S-9) was stored at -80°C and used for the experiments.

Equi-volumes of aqueous solutions of test compound and S-9 were mixed and incubated at 37°C with gentle shaking. After 15, 30, 60, and 120 min, each incubate was boiled for one minute for inactivation of the peptidase. The residual activity of the supernatant obtained by centrifugation at 3,000 x g was determined by the cup diffusinon method using Shigella sonnei EW 33.

Stability of the test compounds against authentic peptidase was examined by the method described in reference.[24] The residual activity was determined by the same manner in the case of S-9.

Pharmacokinetic Studies

Aqueous solutions of test compounds including Alafosfalin were perorally administrated to male mice (dd Y strain of body weight approx. 30 g). At suitable intervals of 5 to 120 min after the dosing, mice were anesthetrized and blood samples were collected by bleeding from the carotid. The blood samples were centrifuged after coagulation at 4°C. The resultant sera were collected and stored at -20°C untill the measurement.

The antibacterial activities of the sera samples were determined by the cup diffusion method using Shigella sonnei EW 33.

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