(TBS) with an excess of calcium carbonate.

In Vitro Antibacterial Assay. Stock solutions of test compounds were prepared at concentrations of 1 mg/100 μL of EtOH or Me_2SO and diluted with growth medium to give threefold dilutions (1 mL) in trypticase soy broth. Test concentrations ranged from 1000 to 0.1 μg/mL. Following inoculation with 50 μL of 10^8 cfu/mL of log phase growth inoculum of *S. mutans*, tubes were incubated at 37 °C in an anaerobic chamber containing an atmosphere of 5% CO_2, 10% H_2, and 85% Ar. Conditions for *A. viscosus* were identical except incubation was aerobic. MIC values were determined visually after incubation 24 h.

In Vitro Antiplaque Assay. Bovine teeth were cut into 4 × 8 mm enamel slabs and sterilized by autoclaving in 0.15 M sodium chloride buffered at a pH of 7.5 with 0.02 M sodium phosphate (PBS). Tooth slabs were coated with sterile human saliva by shaking them in a tight lid dish on a rotating platform at 150 rpm at 37 °C for 1 h. The saliva-coated tooth slabs were rinsed twice in PBS and put in the drug solution for 2 min. The drug solution was removed, and the tooth slabs were rinsed in PBS for 1 min. Each slab was placed in 10 × 75 cotton-stoppered glass culture tubes containing 1 mL of growth medium (supplemented with 1% sucrose) which was inoculated with 50 μL of the plaque-forming organism. Incubation was for 24 h at 37 °C under anaerobic conditions as required. After this period, nonadherent cells were removed by pipetting off the supernatant. Cells adherent to the tooth slabs or to the culture tube were washed twice with 0.5 mL of PBS and the washings combined with previously removed nonadherent cells. In this process, three fractions were obtained: tooth adherent, glass adherent, and nonadherent cells. One mL of 0.1 N NaOH was added to each culture containing tooth slab and glass adherent cells. Cells adherent to tooth slabs were suspended by sonification for 20 s with a sonifier equipped with a microtip. Nonadherent cells were centrifuged at 1500*g* for 20 min at 4 °C. The supernatants were removed with a pipet and the cells were resuspended in 1 mL of PBS. The cells were centrifuged a second time and the pellet resuspended in 1 mL of 0.1 N NaOH. The optical density (OD) of each of the three fractions was read at 540 nm on a Beckman DU-2 spectrophotometer in a cuvette with a 1-cm light path. Cell adherence to tooth slabs and glass is referred to as plaque formation. Controls consisted of tooth slabs immersed for 2 min in sterile distilled water and then incubated in inoculated medium. All experiments were run in triplicate. The combined readings from nonadherent and adherent cells is referred to as total growth. For the compounds reported in this study, effects upon adherence correlated with those upon total growth. Effects upon total growth are reported since these values were more reproducible. Total growth values for the controls represent 100% growth. The IC_{50} values for inhibition of total growth were determined by probit analyses of the inhibition of total growth vs. test concentrations of the compounds. In a typical analysis the standard error in measurement was less than 25% of the mean value obtained in triplicate runs.

Acknowledgment. This research was supported in part by the Periodontal Disease Clinical Research Center at Buffalo, NIDR Grant DE04898, and by NIDR Project Grant DE04744.

Registry No. 2a, 87-10-5; **5**, 98688-41-6; **6**, 98688-42-7; **7**, 98688-43-8; **8**, 98688-44-9; **9**, 98688-45-0; **10**, 98688-46-1; **11**, 98703-76-5; **12**, 98688-47-2; **13**, 98688-48-3; **14**, 98688-49-4; **15**, 98688-50-7; **16**, 98688-51-8; **17**, 98688-52-9; **18**, 98688-53-0; **19**, 98688-54-1; **20**, 98688-55-2; **21**, 98688-56-3; **22**, 98688-57-4; **23**, 98688-58-5; **24**, 98688-59-6; **25**, 98688-60-9; **26**, 98688-61-0; **34**, 17243-13-9; **35**, 19479-88-0; salicylic acid, 69-72-7; 5-(methylsulfonyl)salicylic acid, 68029-77-6; 5-(butylsulfonyl)salicylic acid, 80955-64-2; 5-(*n*-hexylsulfonyl)salicylic acid, 98688-62-1; 5-(*n*-octylsulfonyl)salicylic acid, 98688-63-2; 5-(*n*-decylsulfonyl)salicylic acid, 98688-64-3; 5-(*n*-dodecylsulfonyl)salicylic acid, 98688-65-4; aniline, 62-53-3; 4-bromoaniline, 106-40-1; 4-(trifluoromethyl)-aniline, 455-14-1; 4-nitroaniline, 100-01-6; 3-(trifluoromethyl)-aniline, 98-16-8; 5-(*n*-tetradecylsulfonyl)salicylic acid, 98688-66-5; 4-cyanoaniline, 873-74-5; 4-methoxyaniline, 104-94-9.

Synthesis and Structure–Activity Relationships of Antibacterial Phosphonopeptides Incorporating (1-Aminoethyl)phosphonic Acid and (Aminomethyl)phosphonic Acid

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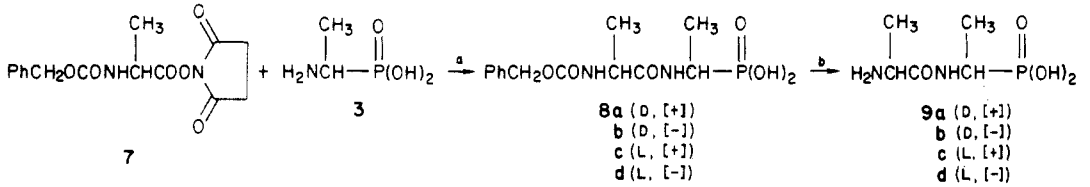
Phosphonodipeptides and phosphonooligopeptides based on L- and D-(1-aminoethyl)phosphonic acids L-Ala(P) and D-Ala(P) and (aminomethyl)phosphonic acid Gly(P) at the acid terminus have been synthesized and investigated as antibacterial agents, which owe their activity to the inhibition of bacterial cell-wall biosynthesis. A method for large-scale synthesis of the potent antibacterial agent L-Ala-L-Ala(P) (**1**, Alafosfalin) is described. Structure–activity relationships in the dipeptide series have been studied by systematic variation of structure **1**. L stereochemistry is generally required for both components. Changes in the L-Ala(P) moiety mostly lead to loss of antibacterial activity, but the phosphonate analogues of L-phenylalanine, L-Phe(P), and L-serine, L-Ser(P), give rise to weakly active L-Ala-L-Phe(P) and L-Ala-L-Ser(P). Replacement of L-Ala in **1** by common and rare amino acids can give rise to more potent in vitro antibacterials such as L-Nva-L-Ala(P) (**45**). Synthetic variation of these more potent dipeptides leads to decreased activity. Phosphonooligopeptides such as $(L\text{-Ala})_2$-L-Ala(P) have a broader in vitro antibacterial spectrum than their phosphonodipeptide precursor, but this is not expressed in vivo, presumably due to rapid metabolism to **1**. Stabilized compounds such as Sar-L-Nva-L-Nva-L-Ala(P) (**46**) have been developed that are more potent in vivo and have a broader in vivo antibacterial spectrum than the parent phosphonodipeptide.

In earlier publications[1-7] we have described the characteristics and the mechanism of antibacterial action of Alafosfalin (**1**) (Alafosfalin is a British Approved Name (B.A.N.) description; it corresponds to earlier names: Ro

(1) Allen, J. G.; Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Holmes, S. W.; Lambert, R. W.; Nisbet, L. J.; Ringrose, P. S. *Nature (London)* **1978**, *272*, 56.
(2) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Lambert, R. W.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1979**, *15*, 677.
(3) Allen, J. G.; Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Holmes, S. W.; Lambert, R. W.; Nisbet, L. J.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1979**, *15*, 684.
(4) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Lambert, R. W.; Lloyd, W. J.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1979**, *15*, 696.

0022-2623/86/1829-0029$01.50/0

Table I. Phosphonopeptide Mimetics (**9**) of Alanylalanine[a]

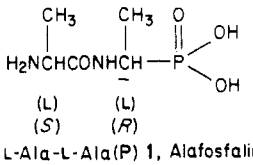


7 3 **8a** (D, [+]) **b** (D, [-]) **c** (L, [+]) **d** (L, [-]) **9a** (D, [+]) **b** (D, [-]) **c** (L, [+]) **d** (L, [-])

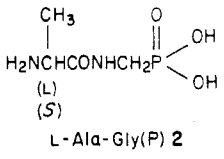
stereochemistry				yield, %		mp, °C (dec)		optical rotation, $[\alpha]^{20}_D$, deg	
7	**3**	**8**	**9**	**8**	**9**	**8**	**9**	**8**[c]	**9**[d]
D	+	D-+	D-+	56	66	223–226	293–294	+36.5	+46.5 (**9a**)
D	−	D-−	D-−	41	68	219–221	295–297	−21.1	−81.1 (**9b**)
L	+	L-+	L-+	41	63	221–223	296–298	+21.0	+84.1 (**9c**)
L	−	L-−	L-−	33	83	229–231	294–295	−34.2	−45.6 (**9d**)[e]
D	(±)	D-+		2		224–226		+33.1	
		D-−		18		219–221		−21.4	
L	(±)	L-+		17		222–223		+21.6	
		L-−		18		228–229		−33.3	

[a] Reagents: (a) $NaHCO_3$, H_2O/EtOH; (b) H_2/Pd/C. [b] Isolated as $PhCH_2NH_2$ salts. [c] *c* 1%, AcOH. [d] *c* 1%, H_2O. [e] Alafosfalin (1).

03-7008, alaphosphalin, and alaphosphin) and various other phosphonopeptides based on (1-aminoethyl)phosphonic acid. Dipeptides such as **2**, which are based on (aminomethyl)phosphonic acid, have been studied to a lesser extent.



L-Ala-L-Ala(P) **1**, Alafosfalin



L-Ala-Gly(P) **2**

This paper describes the methods used for the preparation of phosphonopeptides incorporating a variety of amino acid residues with L and D stereochemistry. The structure–activity relationships for in vitro antibacterial action of those compounds are interpreted in terms of the mechanisms of action deduced in our earlier investigations.

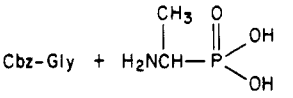
Synthesis of Phosphonodipeptides

At the outset of the investigation we set out to prepare the four diastereoisomers of (alanylaminoethyl)phosphonic acid and the two enantiomeric forms of (alanylaminomethyl)phosphonic acid and those of (glycylaminoethyl)-phosphonic acid as analogues of the bacterial cell-wall component D-alanyl-D-alanine (D-Ala-D-Ala).

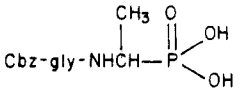
Coupling of dialkyl (aminoalkyl)phosphonates with protected amino acids was not at that time a feasible route since the strong acid conditions needed to deprotect the phosphonate esters would have also hydrolyzed the dipeptide. The free phosphonic acids were therefore coupled with protected amino acids to avoid this difficulty.

Other investigators have subsequently prepared phosphonodipeptides as mixtures of diastereoisomers by coupling "N-terminal"-protected amino acids with racemic diethyl (1-aminoalkyl)phosphonates[8-13] or racemic (1-aminoalkyl)phosphonic acids.[12,13] Separation of free phosphonodipeptide diastereoisomers by ion-exchange chromatography has also been reported.[13-15]

In our initial studies racemic (1-aminoethyl)phosphonic acid (**3a**) was coupled with *N*-carbobenzyloxyglycine (Cbz-Gly) by using the carbonic acid mixed anhydride route. Hydrogenolytic deprotection of **4** gave a dipeptide

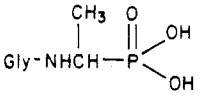


3a (D,L) **b** (D, [+]) **c** (L, [-])



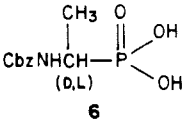
4

that had weak antibacterial activity. The acidic precursor **4** was easily resolved with use of readily available (+)- and (−)-α-methylbenzylamine, to give, after hydrogenolysis, enantiomers **5a** and **5b**. Similar resolution of known (*N*-carbobenzyloxy-1-aminoethyl)phosphonic acid[9,16] (**6**) failed.

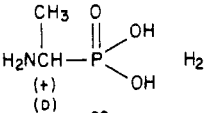


5a, (D, [+]) **b**, (L, [-])

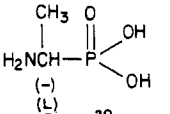
Resolution of **6** was achieved with use of quinine, which formed a salt with the (+) enantiomer. Dehydrobietylamine then formed a salt with the (−) enantiomer. These salts on treatment with base and hydrolgenolysis then gave the previously described[2] enantiomers **3b** and **3c**. The assignment of the stereochemistry shown in terms of L and D and *R*,*S* nomenclature has been described.[2]



6



3b



3c

(5) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Holmes, S. W.; Lambert, R. W.; Lloyd, W. J.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1980**, *18*, 897.
(6) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Holmes, S. W.; Lambert, R. W.; Lloyd, W. J.; Nisbet, L. J.; Ringrose, P. S.; Westmacott, D. *Antimicrob. Agents Chemother.* **1981**, *20*, 470.
(7) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Lambert, R. W.; Lloyd, W. J.; Ringrose, P. S.; Westmacott, D. *Antimicrob. Agents Chemother.* **1982**, *22*, 571.
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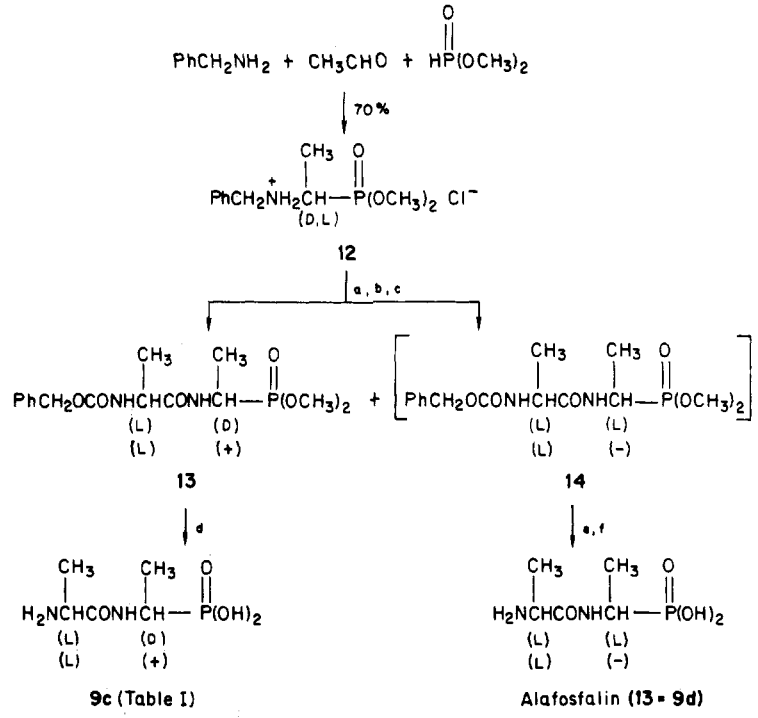
(9) Huber, J. R.; Gilmore, W. F.; Robertson, L. W. *J. Med. Chem.* **1975**, *18*, 106.
(10) Okada, Y.; Iguchi, S.; Mimura, M.; Yagyu, M. *Chem. Pharm. Bull.* **1980**, *28*, 1320.
(11) Kametani, T.; Suzuki, Y.; Kigasawa, K.; Hiiragi, M.; Wakisaka, K.; Sugi, H.; Tanigawa, K.; Fukawa, K.; Orino, S.; et al. *Heterocycles* **1982**, *18*, 295.
(12) Kametani, T. *Heterocycles* **1981**, *16*, 1205.
(13) Kupczyk-Subotkowska, L.; Kafarski, P.; Kowalik, J.; Lejczak, B.; Masterlerz, P.; Oleksyszyn, J.; Szewczyk, J. *ACS Symp. Ser.* **1981**, *171*, 187.
(14) Szewczyk, J.; Lejczak, B.; Kafarski, P. *Experientia* **1982**, *38*, 983.
(15) Kafarski, P.; Lejczak, B.; Mastalerz, P.; Szewczyk, J.; Wasielewski, C. *Can. J. Chem.* **1982**, *60*, 3081.

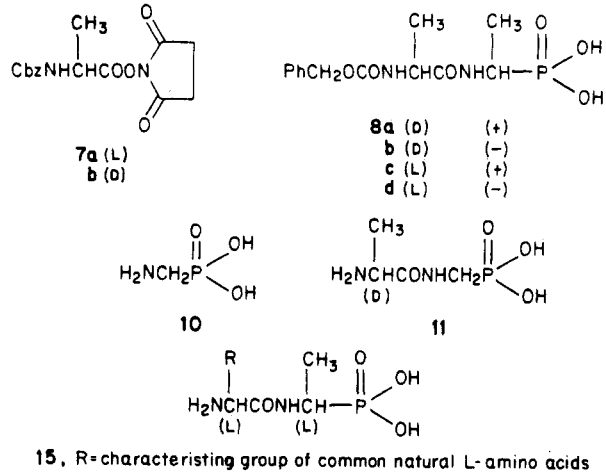
Scheme I. Ester Synthesis of (*N*-L-Alanyl-L-1-aminoethyl)phosphonic Acid [Alafosfalin (1)][a]



[a] Reagents: (a) H_2/Pd/C, (b) $PhCH_2OCONHCH(CH_3)$-COOH (L), DCCI, Et_3N, (c) Et_2O/EtOAc, to crystallize 13, (d) concentrated HCl, (e) HBr/HOAc, (f) propylene oxide, MeOH.

Alternatively, the enantiomers **3b** and **3c** could be conveniently obtained by acid hydrolysis of the phosphonodipeptides **5a** and **5b**.

Coupling of **3c** with the *N*-hydroxysuccinimide ester of *N*-carbobenzyloxy-L-alanine (**7a**, Cbz-L-Ala-ONSu) gave **8d** (L, [-]), which was converted to Alfosfalin (**1**) by hydrogenolysis as shown for the conversion of **8** to **9** in Table I [**9d** is Alafosfalin (**1**)].



The other diastereoisomers of **8d** were similarly prepared and hydrogenolyzed to give (*N*-L-alanyl-D-1-aminoethyl)-phosphonic acid (L, [+]) (**9c**), (*N*-D-alanyl-D-1-aminoethyl)phosphonic acid (D, [+]) (**9a**), and (*N*-D-alanyl-L-1-aminoethyl)phosphonic acid (D, [-]) (**9b**) as shown for **3** + **7** → **8** → **9** (Table I).

In later experiments it was found that when racemic (1-aminoethyl)phosphonic acid (**3a**) was coupled with the *N*-hydroxysuccinimide ester **7a** or its D isomer **7b** (Cbz-D-Ala-ONSu), the resulting diastereomers could be separated very readily by crystallization of the monobenzylamine salts (Table I). This was a more practicable route to **9** than through the resolved acids **3b** and **3c**. Acid hydrolysis of the dipeptides **9c** and **9d** gave **3b** and **3c**, respectively; this was the initial source of the enantiomers for coupling to amino acids other than alanine. Deuterium-labeled compounds were also obtained in this manner.

(Aminoethyl)phosphonic acid (**10**) was similarly converted to the phosphonodipeptides **2** and **11** by using the *N*-hydroxysuccinimide active ester coupling route and hydrogenolytic deprotection.

Mixed anhydride coupling reactions were more capricious and yields varied from the 71% obtained for the synthesis of **4** down to about 20%. This was probably a consequence of the two-phase reaction medium used and the slow coupling reaction compared to normal peptide synthesis. The low yields were improved by using a petroleum ether–aqueous reaction medium with *N*-methylmorpholine instead of triethylamine as base, whereby **8a** was ultimately obtained in 52% yield.

Yields for the coupling reactions shown in Table I were greatly improved by using aqueous dimethylformamide as the reaction solvent with triethylamine as base and yields of up to 87% were obtained.

The synthesis of phosphonopeptides by the ester route was investigated toward avoiding the use of aqueous media. Alafosfalin was prepared most conveniently in this way (Scheme I). Batches exceeding 100 g were readily synthesized in the laboratory. Acid **3c** obtained in high yield by hydrolysis of Alafosfalin was used for free acid coupling experiments with the common L-amino acids to give additional phosphonodipeptides[5] (**15**) which were investigated for antibacterial properties.

Alternatively, acids **3b** and **3c** could be protected[9] (Scheme II) to allow ester couplings[8-13] of, for example, **19** and **22** to give fully protected phosphonodipeptides **21**, which with HBr–AcOH gave phosphonodipeptides **15**. Hydrogenolysis of **21** followed by similar sequential couplings and deprotections gave a wide range of phosphonooligopeptides.[2,5]

Table II summarizes the phosphonodipeptides prepared as a result of systematic structural changes in the substitution pattern. Syntheses were generally carried out by coupling the Cbz-protected *N*-hydroxysuccinimide esters

Scheme II. Protection of the Enantiomers of (1-Aminoethyl)phosphonic Acid

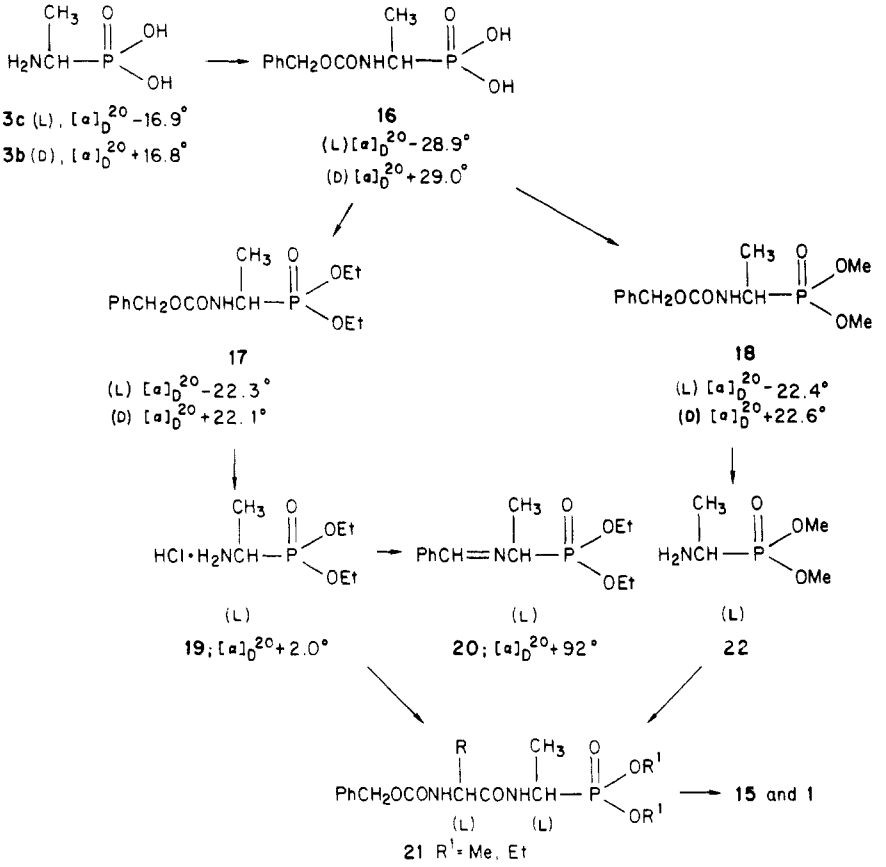
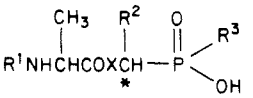


Table II. Phosphonodipeptide Variants of Alafosfalin (1)

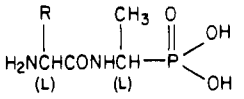


variation, compd	R^1	R^2	R^3	X	optical confign C*	mp, °C (dec)	$[\alpha]^{20}_D$,[a] deg
N-terminus (R^1)							
23	CH_3[b]	CH_3	OH	NH	L	309–310	−56.3
24	CH_3[b]	H	OH	NH	–	295–296	+8.7
25	CH_3CO[c]	CH_3	OH	NH	L	247–249	−86.9
26	HCO[c]	CH_3	OH	NH	L	212–213[d]	−70.7
(1-aminoalkyl)phosphonic acid (R^2)							
27	H	CH_2Cl[e]	OH	NH	DL	ca. 200	+15.3
28a	H	CH_2OH[f]	OH	NH	L	252	−53.0
28b	H	CH_2OH[f]	OH	NH	D	260–263	+84.0
29a	H	$(CH_2)_2CO_2H$[g]	OH	NH	L	190	−3.6
29b	H	$(CH_2)_2CO_2H$[g]	OH	NH	D	195	+44.3
phosphoryl group (R^3)							
30	H	CH_3	OCH_3	NH[h]	L	203–205	−44.2
31	H	H	CH_3	NH	–	ca. 240	+26.9
amide bond (X)							
32	H	H	OH	$NHCH_2$[i]	–	283–284	+17.2
33	H	H	OH	O[j]	–	220–224	
34	H	H	OH	NCH_3	–	195–200	+6.2
35	H	CH_3	OH	NHNH[k]	DL	258–260	−18.5

[a] *c* 1% H_2O; except **23** and **27–29a** where *c* = 0.5% H_2O and **24** where *c* = 0.2% H_2O. [b] From CbzN(Me)-L-Ala.[17] [c] Acylation of 1. [d] $PhCH_2NH_2$ salt. [e] HBr/HOAc deprotection. [f] Diastereoisomers crystallized H_2O/EtOH. Acid hydrolysis gave L-$H_2NCH(CH_2OH)P(O)(OH)_2$, L-Ser(P), $[\alpha]^{20}_D$ −31.2,[a] and D-Ser(P), $[\alpha]^{20}_D$ +32.6.[a] [g] Diastereoisomers separated on cation-exchange resin. [h] LiSCN cleavage of 14. [i] β-Ala-Gly(P), mp 252–253 °C dec, was antibacterially inactive. [j] Gly-O-$CH(CH_3)P(O)(OH)_2$ (DL), mp 269–271 °C dec, was inactive. [k] Cbz-L-AlaNHNH$_2$ and $CH_3COP(O)(OMe)_2$, then dimethyl borane, then Me_3SiCl and HBr/HOAc deprotection.

of amino acids with the free alkylphosphonic acids, followed by hydrogenolysis. Physical constants are also shown. Very little antibacterial activity was found in **23–35**. When it became apparent that for phosphonodipeptides to have good antibacterial activity L,L-stereochemistry was mandatory, alternative common natural L-amino acids were coupled to **3c** with use of *N*-benzyloxycarbonyl protection and *N*-hydroxysuccinimide activation wherever possible. The physical properties and antibacterial activities of these compounds (**15**) have already been reported.[2,5] Variants of **15** in which both rare and also wholly synthetic L-amino acids were attached to

Table III. Chemical Data on New Phosphonodipeptides L-X-L-Ala(P)



15 (L-X-L-Ala(P))

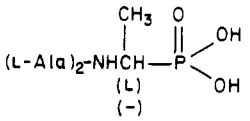
name (X)	R	mp, °C (dec)	$[\alpha]^{20}_D$,[a] deg
Ala(3-F)	CH_2F	240	−30.4[b]
Arg(NO_2)	$(CH_2)_3NHC(NH)NHNO_2$	240–243	−13.2
Arg(homo)	$(CH_2)_4NHC(NH)NH_2$	195	−11.9
Cys(SMe)	CH_2SCH_3	222–224	−32.8[c]
Cys(*n*-Pr)	$CH_2SCH_2CH_2CH_3$	224–226	−25.4[c]
Met(SO)[d]	$CH_2CH_2S(O)CH_3$	256–258	−6.3
Met(SO_2)	$CH_2CH_2S(O_2)CH_3$	255–258	−7.6
2-thienylmethyl		195	−53.7

[a] Specific optical rotation (in degrees of rotation); *c* 0.5% in H_2O unless otherwise stated. [b] *c* 0.2% in H_2O. [c] *c* 1% in 1 N NaOH. [d] DL stereochemistry at S=O.

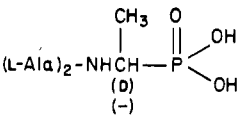
3c were also prepared by using the same general procedures. Physical properties of these new compounds (**15**) are shown in Table III.

Synthesis of Phosphonooligopeptides

Reaction of phosphonodipeptides **9d** and **9c** (Table I) with active ester **7a** followed by deprotection gave tripeptides, such as **36** and **37** in excellent yields. Similar coupling processes gave rise to a variety of phosphonooligopeptides up to the hexapeptide level.[2,5]



36

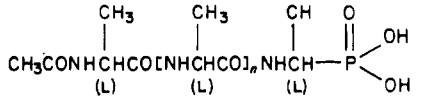


37

Alternatively, phosphonate esters **13** and **14** (and their D,D and D,L analogues, similarly prepared) were hydrogenolyzed and the resulting protected dipeptides were coupled by the ester route (Scheme I) to other N-terminal-protected amino acids. The free phosphonooligopeptides[2] were obtained by HBr/AcOH hydrolysis.

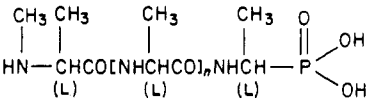
The phosphonotripeptide **36** had a broader in vitro antibacterial spectrum[5] from that of Alafosfalin (**1**) (Table VI). However, this breadth of spectrum was not expressed in vivo, presumably due to rapid breakdown to **1**. Consequently variations at or near the N-terminal amino acid of phosphonooligopeptides, but using the all-alanine series as a model, were investigated in an attempt to improve in vivo stability.

N-Acetyl derivatives **38** and **39** were prepared by conventional routes.



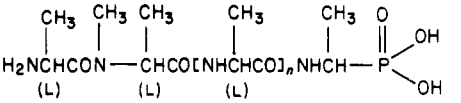
38, *n* = 1
39, *n* = 2

N-Terminal-methyl phosphonodipeptides **23**, **40**, and **41** were prepared by coupling the *N*-hydroxysuccinimide ester of *N*-methyl-*N*-(benzyloxycarbonyl)-L-alanine,[17] prepared in situ, with the appropriate peptide. N-Methyl-substituted amides **42** and **43** and N-terminal sarcosyl peptide **44** were prepared by mixed anhydride coupling, by acti-

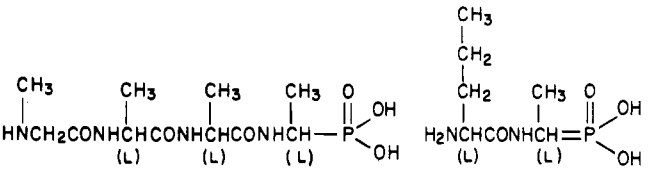


23 *n* = 0
40 *n* = 1
41 *n* = 2

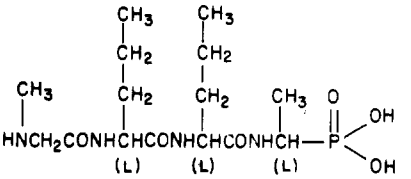
vated ester coupling, and by the alkylation method of Olsen,[17] respectively.



42, *n* = 0
43, *n* = 1

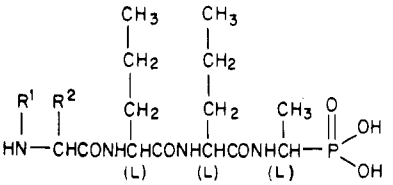


44 **45**



46

Variants **47–50** of sarcosyl peptide **46** were synthesized from **45** via the tripeptide by coupling with the appropriate *N*-hydroxysuccinimide ester, followed by deprotection.



47, R^1=CH_3; R^2=*n*-Pr
48,[5] R^1=CH_3; R^2=$CHMe_2$
49, R^1=CH_3; R^2=CH_2CHMe_2
50,[5] R^1=*n*-Pr; R^2=H

Antibacterial Activity. Phosphonodipeptide variants **23–35** of Alafosfalin (**1**) had very little or no antibacterial activity (Table II). Simple variations of R^2 (Table II) had been shown previously to lead to inactive compounds and only the L-phenylalanine analogue (R^2 = L-CH_2Ph) had significant antibacterial activity.[2] The lack of activity of **27** (R^2 = DL-CH_2Cl) was surprising since L-3-chloroalanine had been reported to be antibacterial.[18] This could have been due to poor transport, lack of cleavage to component amino acids, or rapid reversal of any effect of the L-3-chloroalanine by the valine and isoleucine present in the test medium.[2] The L-alanyl-L-serine phosphonate analogue **28a** (R^2 = L-CH_2OH) (the active diastereoisomer was assigned L,L stereochemistry; the (−) optical rotation was consistent with that of **1** and related phosphonodipeptides[5]) did show weak activity, but this was not found for the L,D diastereoisomer. Interestingly, both the L,L and L,D diastereoisomers of the L-alanylglutamic acid phosphonate analogues **29a** and **29b** [R^2 = $(CH_2)_2COOH$] had a broad spectrum of antibacterial activity in minimal in vitro test media (M. J. Hall, unpublished data); the L,D diastereoisomer was half as active as the LL, compound.

(16) Chambers, J. R.; Isbell, A. F. *J. Org. Chem.* **1964**, *29*, 832.
(17) Olsen, R. K. *J. Org. Chem.* **1970**, *35*, 1912.
(18) Arfin, S. M.; Koziell, D. A. *J. Bacteriol.* **1971**, *105*, 519.

Table IV. Antibacterial Activity of Phosphonodipeptides L-X-L-Ala(P) [X = α-Amino Acid]

N-terminal amino acid in L-X-L-Ala(P),[b] X	antibacterial activity MIC (μg/mL)[a] against: *E. coli* NCIB 8879	*K. aerogenes* 331001	*Enterobacter* 250002	*S. marcescens* ATCC 14756	*S. typhimurium* 538003	*H. influenzae* NCTC 4560	*S. faecalis* 58511	*S. aureus* NCIB 8625
natural								
Ala	1	0.5	1	8	64	64	2	32
Arg	0.25	<0.12	1	2	32	128	16	16
Cys	32	16	i[c]	i	i	i	i	i
Met	0.25	0.12	1	2	8	16	1	1
Phe	0.5	1	2	4	32	32	2	4
L-Nva	0.03	0.015	0.25	0.5	0.25	4	1	8
synthetic (see Table III)								
Ala(F)	0.25	4[d]	32[e]	i[f]	32	8	32	16
Arg(NO_2)	0.5	2	8	4	64	NT[g]	2	32
Arg(homo)	0.25	0.12	2	2	2	64	2	128
Cys(SMe)	0.03	0.03	0.03	0.5	0.25	4[h]	2	16
Cys(S-*n*-Pr)	0.06	0.03	0.06	0.5	0.25	64[h]	2	8
Met(SO)[i]	4	4	32	32	i	16	4	8
Met(SO_2)	16	32	i	i	i	32	4	32
2-thienylmethyl[j]	0.5	0.25	1	4	4	32[k]	2	32

[a] MIC = minimum inhibitory concentration. [b] Abbreviations: Ala = alanyl, Arg = arginyl, Cys = cysteinyl, Met = methionyl, Phe = phenylalanyl, L-Nva = norvalyl; others, see Table III. [c] i = MIC > 128 μg/mL. [d] Strain OGKA-1. [e] Strain 1/9 Ba. [f] Strain ATCC 7700. [g] NT = not tested. [h] Strain H1. [i] DL stereochemistry at SO. [j] DL-amino acid. [k] Strain 314041.

The antibacterial activity of L-Ala-D-Glu(P) could be due to its substitution for L-Ala-D-Glu in the synthesis of the muramyl pentapeptide of the peptidoglycan in the bacterial cell wall. These compounds did not have antibacterial activity in defined test media[2] and in vivo.

Replacement of the alanyl moiety in the L-Ala-L-Ala(P) and L-Ala-Gly(P) free dipeptide series, to give compounds such as **15**, which contain common natural L-amino acids other than L-alanine, led to the identification of a large number of antibacterial compounds.[2,5,7] Selected examples are shown in Table IV.

When derivatives of natural L-amino acid residues (Table III) were substituted for L-alanine, there was no significant improvement in in vitro antibacterial activity (Table IV). (In this context the cysteinyl derivatives Cys(SMe) and Cys(S-*n*-Pr) are regarded as lipophilic analogues of the rare L-amino acid L-norvaline.) Others[11] have reportes similar conclusions for the in vivo antibacterial activity of analogues of Alafosfalin.

Although the *N*-acetyl derivative **25** of the phosphonodipeptide alafosfalin (**1**) was inactive in vitro, the *N*-acetyl tripeptide **38** and tetrapeptide **39** had activity against *Escherichia coli*, in vitro similar to **1**. However **38** was inactive in vivo orally and subcutaneously (sc) whereas **39** was equiactive with **1** orally but was inactive subcutaneously. This result was attributed to rapid oral route breakdown of **39** to orally active **1**.

Structure–activity relationships for the active transport of dipeptides into bacteria had indicated that terminal *N*-methyl derivatives were acceptable substrates.[19b] However, in the phosphonodipeptide series, **23** and **24** had little or no activity; the related *N*-methyl tripeptide **40** had modest in vitro activity. The *N*-methyl tetrapeptide **41** was as potent as **1** in vitro but had a narrower antibacterial spectrum; it was about half as active as **1** in vivo against *E. coli*, orally and subcutaneously.

The amide *N*-methyl tripeptide **42** and the corresponding tetrapeptride **43** had reduced activities in vitro.

Table V. Chemical Data on New Phosphonooligopeptides Y-(L-X)(L-Z)-L-Ala(P)[a]

Y-(L-X)(L-Z)	mp, °C (dec)	$[\alpha]^{20}_D$, deg
Sar-Ala-Arg	~205	−78.8[b]
Sar-Ala-Nva	291–292	−110[c]
(*N*-Me)Nva-Nva_2 (**47**)	307–309	−81.1[d]
(*N*-Me)Leu-Nva_2 (**49**)	317–318	−39.9[e]

[a] X and Z = amino acid; Y = N-terminally modified amino acid. [b] *c* 0.5% in H_2O. [c] *c* 0.25% in H_2O. [d] *c* 0.5% in 0.1 N NaOH. [e] *c* 0.5% in CF_3COOH.

These studies suggested that phosphonotripeptides which had been modified to reduce the rate of hydrolysis in vivo were of little value, but stabilized phosphonotetrapeptides, e.g., **41**, could have potential for therapy. Other *N*-methyl amino acids[20] were investigated as alternatives to the *N*-methylalanyl moiety of **41**. The sarcosyl peptide **44** had the characteristic phosphonooligopeptide activity against *Streptococcus faecalis* and *Haemophilus influenzae* (Table VI). Compound **44** was also significantly more potent than **1** against *E. coli* in vitro (Table VI) and was about 8 times more active in vivo against *E. coli* subcutaneously than **1** on a molar basis (Table VI). Compound **44** had interesting in vitro antibacterial activity against several strains of the respiratory tract pathogens *H. influenzae*, *Streptococcus pneumonia*, and *Streptococcus pyogenes* whereas Alafosfalin (**1**) was almost inactive against these organisms.[5] In vitro activity was not improved when the *N*-methyl group of **44** was replaced by alternative alkyl residues.[21]

In the course of later studies, several more potent (in vitro) phosphonodipeptides than alafosfalin (**1**) were identified; of these compounds, **45**, L-Nva-L-Ala(P),[2,5] was exceptional. Consequently a range of related sarcosyl phosphonotripeptides was synthesized[22] (Table V). Relative molar in vivo activities againt *E. coli* of representative sarcosyl tripeptides, compared to parent phosphonodipeptides, are shown in Table VI. These results

(19) Payne, J. W. In "Peptide Transport in Protein Nutrition"; Matthews, D. M., Payne, J. W., Ed.; North-Holland Elsevier: Amsterdam, 1975, (a) Chapter 10, "Microbial peptidohydrolases", p 367, (b) Chapter 9, "Transport of peptides in micro-organisms", p 337.

(20) Greenstein, J. P.; Winitz, M. "Chemistry of the Amino Acids"; Wiley: New York, 1961; pp 22, 33, 1415.

(21) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Lambert, R. W.; Lloyd, W. J.; Lord, A. V.; Ringrose, P. S.; Westmacott, D. *Antimicrob. Agents Chemother.* **1983**, *24*, 552.

(22) United Kingdom Patent GB 2030148B.

Table VI. Comparison of Antibacterial Activity of Phosphonodipeptides and Phosphonooligopeptides in Vitro and in Vivo

Y-(L-X)$_m$(L-Z)$_n$-L-Ala(P)[a] where N-terminal amino acid or peptide is:	in vitro antibacterial activity MIC (μg/mL)[b] against:				in vivo activity, molar CD_{50} rel to 1, L-Ala-L-Ala(P)[c-f] against *E. coli* 281007	
	E. coli NCIB 8879	*S. faecalis* FS5	*S. aureus* 561037	*H. influenzae* NCTC 4560	po	sc
Ala (Alafosfalin, **1**)	0.5	2	4	4	1.0	1.0
Arg	0.25	16[g]	16[h]	128	7.7	8.3
Nva (**45**)	0.03	1[g]	8[h]	4	1.1	8.8
Ala_2 (**36**)	0.5	0.06	4	0.06		
Ala_3	0.12	0.25	32	0.06		
Sar-Ala_2 (**44**)	0.016	0.06	>128	0.03	1.05	8.0
Sar-Nva_2 (**46**)	0.04	0.12	16	0.015	1.1	19
(*N*-Me)Nva-$(Nva)_2$ (**47**)	0.5	0.06	8	0.25		
(*N*-Me)Val-$(Nva)_2$ (**48**)	16	8	>128	0.25[i]		
(*N*-Me)Leu-$(Nva)_2$ (**49**)	2	0.25	32	0.25[i]		
Sar-Ala-Arg	≤0.007[j]	2	>128	2.0	1.1	13.5
Sar-Ala-Nva	≤0.007[j]	0.12	32	0.06	1.0	18

[a] X, Z = amino acid; Y = amino acid or N-terminally modified amino acid. [b] MIC = minimum inhibitory concentration. [c] Mouse septicaemia model. [d] Dosed 1, 3, and 5 h postinfections with *E. coli* 281007. [e] Relative molar activity of analogue, both in μmol/kg. [f] Range of CD_{50} for L-Ala-L-Ala(P) = 10–100 mg kg^{-1} po and 3–10 mg kg^{-1} sc. [g] Strain 585011. [h] Strain NCIB 3625. [i] Average MIC against six strains of *H. influenzae*. [j] Strain 281007.

demonstrate that the improved in vitro activity of phosphonooligopeptides can be expressed in vivo (subcutaneously), e.g., **44** compared to **1** and **46** compared to **45**. In vivo activity does not arise solely from rapid N-terminal metabolism to the active parent phosphonodipeptide. Compound **46** was selected for further study. It had in vivo activity[5] against the selected respiratory tract pathogens. The *N*-methyl-L-norvalyl (**47**), *N*-methyl-L-valyl[5] (**48**), *N*-methyl-L-leucyl (**49**), and *N*-*n*-propylglycyl[5] (**50**) analogues of **46** were significantly less active in vitro (Table VI) and in vivo.[5]

Discussion of Structure-Activity Relationships

Early results[1,2] showed that only the L stereochemistry (Table I) was acceptable for antibacterial activity in phosphonodipeptide mimetics of alanylalanine. Mechanistic studies showed that **1** and **2** were transported into bacteria by L-specific permeases and cleaved intracellularly by L-specific peptidases to Ala(P) (**3c**) and Gly(P) (**10**), which inhibit alanine racemase.[4,7] Variation of the Ala(P) (**3c**) moiety of phosphonodipeptides led to loss of antibacterial activity.[2] Only close mimetics of alanine were good substrates for alanine racemase. Dipeptides based on Gly(P) were invariably less potent in vitro than those based on Ala(P) (**3c**).[7]

More recently, Cheung[23] and co-workers, in a study of chloroalanyl and propargylglycyl dipeptides, also report the restriction of antibacterial activity to compounds having L,L stereochemistry. In this case, β-chloro-L-alanine is known from work of Wang and Walsh to be a substrate for *Escherichia coli* alanine racemase.[24] Further derivatives of alanine that inhibit alanine racemase and other enzymes involved in peptidoglycan biosynthesis have been reviewed by Neuhaus and Hammes.[25]

Differences in in vitro antibacterial activity have previously been explained in terms of varying degrees of transport into bacterial cells, followed by intracellular hydrolysis to release the active entity L-Ala(P) or Gly(P).[1,2,3,5,7,21] In the series of antibacterially inactive phosphonodipeptides,[2] L-Ala-NHCH(R)P(O)$(OH)_2$ (R ≠ CH_3 or H), uptake into *E. coli* was markedly reduced for L,L diastereoisomers and absent for L,D compounds. Intracellular hydrolysis of these accumulated L,L dipeptides was about half that found for Alafosfalin (**1**) and L-Ala-Gly(P) (**2**). In turn, the rates of hydrolysis of **1** and **2** by *E. coli* were less than one-tenth of that found for their all natural L-amino acid counterparts L-Ala-L-Ala and L-Ala-Gly (P. S. Ringrose and W. J. Lloyd, unpublished data).

The lack of antibacterial activity of other variants of alafosfalin (**1**) (Table II) may now be explained by their failure to be transported into bacteria by L,L dipeptide permeases.[21]

Of the dipeptides based on phosphonate analogues of natural amino acids, the phenylalanine,[2] Phe(P), serine, Ser(P) (**28**), and glutamic acid, Glu(P) (**29**), compounds (Table II) showed weak antibacterial activity, but the derivative of valine,[2] Val(P), was inactive. Glu(P) could act in a different way on bacterial peptidoglycan biosynthesis by virtue of the role of glutamic acid in the bacterial cell wall.[4] This agrees with the exceptional finding of antibacterial activity for both L-Ala-L-Glu(P) and L-Ala-D-Glu(P) (this is the only example of an antibacterially active phosphonodipeptide incorporating a D residue), the latter being an analogue of the L-Ala-D-Glu sequence in the bacterial cell wall.[4]

Replacement of one hydroxyl group of the phosphonic residue (Table II) by methyl gave compound **31** with substantially less activity than for **2**.

The antibacterial inactivity of amide bond variants **32–35** of phosphonodipeptides (Table II, X ≠ NH) may be interpreted in terms of transport and hydrolysis requirements. The *N*-methyl amide **34** (Table II) meets the structural requirements for peptide transport in bacteria,[19b] but the lack of activity may be explained in terms of resistance to intracellular hydrolysis and poor alanine racemase inhibitory activity of any (*N*-Me)-Gly(P) released.

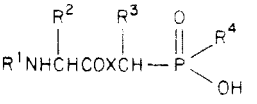
Simple terminal *N*-acyl dipeptides are not transported into bacteria.[19b] The lack of antibacterial activity found for *N*-acyl phosphonodipeptides **25** and **26** may also be due to their failure to be transported but could also be due to their failure to be hydrolyzed intracellularly. In contrast, terminal *N*-alkyl dipeptides are known to be transported into bacteria.[19b] The absence of antibacterial activity found for *N*-alkyl phosphonodipeptides **23** and **24** may be

(23) Cheung, K.-S.; Wasserman, S. A.; Dudek, E.; Lerner, S. A.; Johnston, M. *J. Med. Chem.* **1983**, *26*, 1733.
(24) (a) Wang, E.; Walsh, C. *Biochemistry* **1978**, *17*, 1313; (b) Wang, E.; Walsh, C. *Biochemistry* **1981**, *20*, 7539.
(25) Neuhaus, F. C.; Hammes, W. P. *Pharmacol. Ther.* **1981**, *14*, 265.

due solely to poor intracellular hydrolysis.

Replacement of natural L-amino acid moieties in **15** by closely related synthetic derivatives or analogues (Table III) produced antibacterially active compounds (Table IV), but in no case was there a significant improvement on the parent amino acid. The *S*-methylcysteinyl derivative (Tables III and IV) had comparable activity to its L-norvalyl analogue **45** in vitro but was markedly less active in vivo. This result may be attributed to more facile mammalian peptidase cleavage of the *S*-methylcysteinyl derivative compared to **45** in vivo.

Structural requirements for significant in vitro antibacterial activity in phosphonodipeptides and their simple analogues are summarized in structure **51** as follows. (i)



51

R^1 is L-amino acid or all-L peptide but not simple acyl or alkyl. (ii) R^2 is a group characteristic of common and rare amino acids and their chemically modified analogues. (iii) L Stereochemistry is required for both amino acid components. (iv) The α-amide bond is essential and cannot be methylated. (v) R^3 is CH_3 or H for good activity, but slight activity is found for the characterising groups of amino acids closely related to alanine. (vi) R^4 is OH, methyl, or H,[26] but not ester. (vii) The free OH group on phosphorus is essential.

Phosphonooligopeptides such as **36** (**37** was almost inactive[2]) are generally much more active than their dipeptide precursor **1** against *Strep. faecalis* and *H. influenzae* (Table VI) and other respiratory tract pathogens in vitro; they are no more active than the parent dipeptide in vivo, presumably due to rapid N-terminal degradation by mammalian peptidases.[5] For potent in vitro antibacterial activity all-L stereochemistry is required up to the hexapeptide level. Alternatives to free amino in the N-terminal function of the phosphonooligopeptides gave rise to advantages in the in vitro antibacterial spectrum in particular cases, but these advantages were not regularly sustained in vivo. The N-terminal sarcosyl residue favored good in vitro activities and in the case of the phosphonotripeptide Sar-Nva-Nva-Ala(P) (**46**) this extended into the in vivo[5] characteristics. The intrinsic nature of this in vivo activity of sarcosyl phosphonotripeptides is shown in Table VI. The sarcosyl derivative **44** was 8 times as active subcutaneously as the parent compound alafosfalin (**1**) in vivo against *E. coli*. Other sarcosyl derivatives were also more active in vivo against *E. coli* than their Arg-Ala(P) and Nva-Ala(P) precursors.

In the case of the sarcosyl tripeptide Sar-Nva-Nva-Ala(P) (**46**) a combination with cephalexin showed a synergistic in vivo effect against *Pseudomonas aeruginosa* in the mouse. This was attributed to release of the dipeptide Nva-Ala(P) since the intact oligopeptide is inactive against this organism in vitro (unpublished data).

Alafosfalin (**1**) is well absorbed orally in humans and tolerance is exceptionally good.[27,28] Clinical trials have shown that the drug is at least as effective as standard agents for the treatment of acute bacterial enterocolitis and shigellosis.[29]

The pharmacokinetics of alafosfalin and cephalexin are well matched,[28] and this combination in envisaged as an effective treatment of urinary tract infections.[6,29] Although the antibacterial characteristics of Alafosfalin favor its use in therapy for Gram-negative organisms, the results described in this paper indicate that there is scope for phosphonopeptides such as Sar-L-Nva-L-Nva-L-Ala(P) (**46**) being used either alone or in combination[6] for applications which require a broader antibacterial spectrum.

Experimental Section

Melting points were determined in open capillary tubes on a Büchi apparatus and are uncorrected. Elemental analyses were carried out on a Perkin-Elmer Model 240 instrument. Analyses are reported only by the elemental symbols and results were within ±0.4% of the theoretical values. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter. Infrared (IR) spectra were determined on a Pye-Unicam SP 1000 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian T-60 or XL 100/15 spectrometer and chemical shifts are presented in ppm from internal tetramethylsilane as a standard.

Homogeneity of the products was determined by ascending thin-layer chromatography (TLC) on precoated cellulose sheets (Eastman Chromatogram Sheet, 13254 Cellulose with fluorescent Inidcator [No. 6065]) using principally the solvent system 1-butanol–acetic acid–water (12:3:5). Products were visualized by successive exposure (where appropriate) to ultraviolet (UV) light, iodine vapor, ninhydrin, and ammonium molybdate spray reagents. The cation-exchange resin used throughout was British Drug Houses, Zerolit 225, SRC 13, RSO_3H, freshly regenerated in the acid cycle.

(*N*-L-Alanyl-L-1-aminoethyl)phosphonic Acid (1, Alafosfalin). Method a. By Separation of Diastereoisomers of N-Protected Phosphonopeptides (8), Table I). A slurry of (DL-1-aminoethyl)phosphonic acid (**3a**, 6.25 g, 50 mmol) in 12.5 mL of H_2O was stirred as triethylamine (10 g, 14 mL, 100 mmol) was added to give a clear solution. Dimethylformamide (25 mL) was added and the solution was cooled to 0 °C and **7a**[30] (19 g, 60 mmol) was added. The resulting thick slurry was stirred at 0 °C for 0.5 h when an almost clear solution was seen and then at room temperature overnight.

The solution was evaporated in vacuo to give a gum which was dissolved in a mixture of 30 mL of H_2O and 60 mL of EtOH and the solution was passed over cation-exchange resin (RSO_3H) which was eluted with the same solvent. An acid fraction of 200 mL was evaporated to give a gum which was dissolved in 100 mL of water and extracted twice with 100 mL of Et_2O. The aqueous was diluted to 300 mL and titrated to pH 4.5 with 4 N aqueous benzylamine. The resulting solid was filtered off and washed with H_2O, EtOH, and Et_2O to give 7.5 g of salt, mp 211–214 °C dec, $[\alpha]^{20}_D$ +17.2° (*c* 1%, AcOH). Recrystallization from 125 mL of H_2O gave 5.9 g (27% based on **3a**) of pure unwanted L,D-diastereoisomer **8c**, mp 223–225 °C dec, $[\alpha]^{20}_D$ +20.5° (*c* 1%, AcOH). The combined filtratres were evaporated (total yield of salt, 87%). This residue was stirred with MeOH (15 mL/g) to give 2.5 g of **8d**, mp 231–233 °C dec, $[\alpha]^{20}_D$ −31.7° (*c* 1%, AcOH). The filtrate was evaporated and stirred with H_2O (25 mL/g) to give 2.1 g of **8c**, mp 216–218 °C dec, $[\alpha]^{20}_D$ +20.7°. The filtrate was evaporated and the residue was stirred with MeOH (15 mL/g) to give 5.1 g of **8d**, mp 225–227 °C dec, $[\alpha]^{20}_D$ −33.1°. Total yield of pure **8d** was 7.6 g (35% based on **3a**).

Use of the 2,4,5-trichlorophenyl ester of *N*-(benzyloxycarbonyl)-L-alanine[31] instead of **7a** in the above reaction gave an 81% yield of diastereoisomeric monobenzylamine salts **8c** and **8d** from which 38% (based on **3a**) of the desired L,L-diastereoisomer **8d** was isolated by the procedures described above.

The L,L diastereoisomer **8d** (4.4 g, 10 mmol) was added portionwise with stirring to 7.5 mL of 45% HBr in acetic acid and

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washed in with 2.5 mL of glacial AcOH. The mixture was stirred for 2 h and then 50 mL of Et_2O was added to precipitate a gum. The ether was decanted and the gum was washed by decantation with 50 mL and then 25 mL of Et_2O. The residual gum was dissolved in 20 mL of MeOH and a solution of 1.5 mL of propylene oxide in 2.5 mL of MeOH was added. The solid precipitate was stood for 2 h at room temperature and for 1 h at 0 °C and then filtered off to give 2.2 g (97%) of the methanolate of **9d** [Alafosfalin (**1**)], mp ca. 290 °C dec. Recrystallization from a mixture of 7 mL of H_2O and 12 mL of EtOH gave 1.6 g (83%) (yields of up to 98% have been obtained on a larger scale) of pure alafosfalin (**1 = 9d**): mp 293–295 °C dec; $[\alpha]^{20}_D$ −45.6°, $[\alpha]^{20}_{365}$ −172° (*c* 1%, H_2O); IR (Nujol) 1657 (amide) cm^{-1}; NMR (D_2O) δ 1.3 (dd, 3, CH_3CHP), 1.6 (d, 3, CH_3CHCO), 3.78–4.38 (m, 2, CH_3CHCO and NC*H*P). Anal. ($C_5H_{13}N_2O_4P$) C, H, N, P.

Phosphonopeptide **1** (7.4 g, 38 mmol) prepared by this route was dissolved in 80 mL of 5 N HCl and refluxed for 2.5 h. Workup using cation-exchange resin separation gave 4.2 g, mp 293–297 °C dec. Recrystallization from a mixture of 25 mL of H_2O and 30 mL of EtOH gave 3.8 g of pure **3c**, mp 296–297 °C dec, $[\alpha]^{20}_D$ −16.5° (*c* 2%, 1 N NaOH) (lit.[2] $[\alpha^{20}_D]$ −16.9°).

Deuterio 1. Deuterium-labeled **3a**, (DL-1-aminoethyl-*1-d*)-phosphonic acid, was prepared from racemic **20** (54 g, 0.2 mol) by reaction at −60 °C in toluene with *n*-butyllithium (95 mL of 27% by weight in hexane; 2.5 molar; 0.24 mol) for 3 h followed by quenching at −10 °C with 10 mL of deuterium oxide and then hydrolysis with concentrated HCl at reflux for 24 h. Coupling of this deuterio **3a** (6.3 g, 50 mmol) with **7a** (19 g, 50 mmol) followed by separation of diastereoisomers as above gave deuterio **8d** and deuterio **8c**. Deprotection of deuterio **8d** (L,L) (7.2 g, 16 mmol) as above gave 2.7 g (86%) of (*N*-L-alanyl-L-1-aminoethyl-*1-d*)phosphonic acid, deuterio **1**, mp 286–288 °C dec, $[\alpha]^{20}_D$ −44.9°.

Deuterium labeled **3c**, (L-1-aminoethyl-*1-d*)phosphonic acid, was prepared by hydrolysis of deuterio **1**, as above.

Method b. By the Ester Route by Separation of Fully Protected Phosphonopeptide Diastereoisomers 13 and 14 (Scheme I). To a solution of benzylamine (600 mL, 5.5 mol) in 600 mL of water at 0 °C was added a solution of acetaldehyde (280 mL, 5.0 mol) during 0.5 h. Dimethyl phosphite (520 mL, 5.65 mol), precooled to −5 °C was added during 0.5 h. The mixture was stirred at 0 °C overnight and then 350 g of NaCl was added and the pH was adjusted to 2–3 by addition of 457 mL of concentrated HCl. Chloroform (1.25 L) was added and the mixture was stirred for 15 min. Phases were separated, and the aqueous layer was reextracted with 1.25 and 0.625 L of $CHCl_3$. The combined solvent phases were dried (Na_2SO_4) and concentrated to 1.9 L. Ethyl acetate (1 L) was added and the volume was again reduced to 1.9 L. This EtOAc procedure was repeated to remove most of the $CHCl_3$ without evaporation to dryness of a labile product. Acetone (2.5 L) was added and the crystallization was completed by storage at 4 °C overnight. The product was filtered off and washed twice with 500 mL of Me_2CO to give 927 g of crude **12**, mp 106–107.5 °C dec, which by NMR contained about 7% of benzylamine hydrochloride. Compound **12** was dissolved in 2.1 L of CH_2Cl_2 and 28 g of benzylamine hydrochloride, mp 252–255 °C, was filtered off. The filtrate was concentrated to 1.1 L and reconcentrated similarly with two separate portions of 700 mL of ethyl acetate. Acetone (2 L) was added and the mixture was stored overnight at 4 °C. The separated solid was filtered off and washed twice with 300 mL of Me_2CO to give 814 g (58%) of pure **12** [purity was assessed by TLC on Merck Kieselgel F_{254} plates developed with pyridine (5%)–MeOH (35%)–benzene (60%) and visualized with iodine (R_f 0.7)], mp 104–106 °C dec. The original mother liquors (from 927 g) were evaporated to give 627 g of gum which was partitioned between 300 g of K_2CO_3 in 600 mL of H_2O and 400 mL of toluene. The solvent extract was dried (Na_2SO_4) and treated with 150 mL of 8 N methanolic HCl and then 600 mL of Me_2CO to give a further 193 g of crude **12**. Purification as above gave a further 170 g (12%) of pure **12**, mp 103–105 °C dec; total yield 984 g (70%).

To 2 L of CH_2Cl_2 was added recrystallized **12** (559 g, 2.0 mol) and 70 g of charcoal. The mixture was hydrogenated in the presence of 60 g of 10% palladium on charcoal catalyst at room temperature and pressure for 3 h. Catalyst was filtered off and *N*-(benzyloxycarbonyl)-L-alanine (446 g, 2.0 mol) was added.

The mixture was cooled to −20 °C (Me_2CO/solid CO_2) and Et_3N (280 mL, 2.0 mol) was added below −10 °C. The temperature was adjusted to 0 °C and a solution of dicyclohexylcarbodiimide (466 g, 2.0 mol) in 200 mL of CH_2Cl_2 was added at 0–2 °C during 1 h. The mixture was placed on an ice bath and stirred overnight. Separated dicyclohexylurea was filtered off and washed twice with 500 mL of CH_2Cl_2. The first filtrate was washed with 1 L of water which was back-extracted with the CH_2Cl_2 washes. The first solvent phase was washed with a solution of 200 g of K_2CO_3 in 1 L of H_2O which was back-washed with the CH_2Cl_2 washes. The combined CH_2Cl_2 extracts were dried (100 g, Na_2SO_4) and concentrated in vacuo at a bath temperature of 30–35 °C and reevaporated twice with 250 mL of EtOAc and then treated with 1.25 L of dry Et_2O and 125 mL of EtOAc and stored for 4 days at 4 °C. The crystalline material was filtered off and washed with a mixture of 20 mL of EtOAc and 180 mL of anhydrous Et_2O and dried to give 299 g (83%) of **13**, mp 131–133 °C [raised to 133.5–135 °C by recrystallization from MeOH (1.4 mL/g) and Et_2O (4 mL/g)]. Anal. ($C_{15}H_{23}N_2O_6P$) C, H, N, P.

The combined filtrate was evaporated to an oil (largely **14**) which was transferred using 75 mL of glacial acetic acid to a 5-L vessel. To it was added 250 mL of 45% HBr in glacial acetic acid in one portion, followed by a further 750 mL during 0.5 h, with external cooling to maintain the temperature below 25 °C. The mixture was then stirred for 5 h at room temperature. Anhydrous ether (3 L) was added and the mixture was stirred for 5 min and allowed to settle and the upper phase decanted. The process was repeated twice with 1 L of ether. The mobile residue was dissolved in 2 L of MeOH and stirred with ice–water cooling as a solution of 280 mL of propylene oxide in 280 mL of MeOH was added during 15 min, the temperature being maintained below 20 °C. A solid was precipitated and the pH of the solution rose to pH 4–5 (moist paper). After stirring for 30 min, the mixture was stored at 4 °C overnight. The resulting granular solid was filtered off and washed with 200 mL of MeOH and dried to give crude **1** as the monomethanolate (NMR), mp 287–288 °C dec, $[\alpha]^{20}_D$ −34.3° (*c* 1%, H_2O). This material was recrystallized by dissolving in 850 mL of water and stirring for 5 min with 4 g of activated carbon which was then filtered and washed with 50 mL of water. The combined filtrates were stirred at room temperature, and 1.8 L of EtOH was added rapidly when crystallization occurred. The mixture was stored at 4 °C overnight and then filtered and the solid was washed with 400 mL of 75% EtOH and then with 400 mL of EtOH and dried in vacuo at 40 °C to give 125.5 g (64%) of pure **1** (Alafosfalin), mp 294–295.5 °C dec, $[\alpha]^{20}_D$ −46.3° (*c* 1%, H_2O). Anal. ($C_5H_{13}N_2O_4P$) C, H, N. The filtrates were evaporated, and the residue was crystallized from a mixture of 200 mL of water and 600 mL of EtOH to give a further 9.55 g (4.9%) of **1**, mp 295–296 °C dec, $[\alpha]^{20}_D$ −43.3° (*c* 1%, H_2O); total yield 135 g (69%).

(D-1-Aminoethyl)phosphonic Acid (3b). For large-scale synthesis of **3b** the fully protected dipeptide **13** (452 g, 1.26 mol), prepared above (as a byproduct in the synthesis of **1** by the ester route, Scheme I), was dissolved in 2 L of CH_2Cl_2 and stirred with 1 L of concentrated HCl, SG 1.18, for 24 h to remove both protecting groups. The phases were separated, and the CH_2Cl_2 layer was washed with 500 mL of H_2O.

The combined aqueous phases (containing **9c**, L,D, Table I) were heated to reflux under a Dean and Stark separator (to remove CH_2Cl_2 and attain the necessary temperature of 106 °C needed to effect the hydrolysis) for 24 h. The solution was evaporated in vacuo and the residue was reevaporated with 250 mL of EtOH. The residue was dissolved in 750 mL of MeOH and cooled to 0–5 °C. Thionyl chloride (125 mL) was added dropwise at 0–5 °C and the mixture was then refluxed for 5 h. The mixture was evaporated in vacuo and reevaporated twice with 100 mL of MeOH. The residue was dissolved in 625 mL of MeOH and propylene oxide (130 mL) was added at room temperature. The mixutre was stirred overnight and the separated solid was filtered off and washed twice with 100 mL of MeOH to give 150 g (95%) of **3b**, mp 294–295 °C dec. Recrystallization from 750 mL of hot water by addition of 1.9 L of EtOH gave 137 g of pure **3b**, mp 295–296 °C dec, $[\alpha]^{20}_D$ +15.4° (*c* 2%, 1 N NaOH) (single spot on TLC). Anal. ($C_2H_8NO_3P$) C, H, N, P.

[DL-1-[(*N*-Carbobenzyloxyglycyl)amino]ethyl]phosphonic Acid (4). A mixture of Et_3N (20 g, 200 mmol) and *N*-carbo-

benzyloxyglycine (42 g, 200 mmol) in 2 L of toluene was cooled to −5 °C. Isobutyl chloroformate (27 g, 200 mmol) was added dropwise followed by a solution of **3a** (6.25 g, 50 mmol) in 50 mL of 2 N NaOH. The mixture was stirred for 5 h at −5 °C and then overnight at room temperature. The aqueous layer was adjusted to pH 9.5 with 2 N NaOH and evaporated to remove Et_3N. The residue was partitioned between H_2O and $CHCl_3$. The aqueous extract was adjusted to pH 2.5 with 2 N HCl and extracted with Et_2O and then with $CHCl_3$. The aqueous extract was passed down a column of cation-exchange resin and the acid eluate was titrated to pH 4 with 1 N aqueous benzylamine. The resulting solid was filtered off and washed with H_2O, EtOH, and Et_2O to give 12 g of pure monobenzylamine salt of **4**, mp 204–206 °C dec. Concentration of filtrates gave a further 3.3 g, mp 205–207 °C dec; total yield 15.3 g (71%). Anal. ($C_{19}H_{26}N_3O_6P$) C, H, N, P.

Resolution of 4. The monobenzylamine salt of **4** (2.1 g, 5 mmol) was converted to the free acid **4** by cation exchange. Titration with (+)-α-methylbenzylamine to pH 4, evaporation, and crystallization from a mixture of 10 mL of MeOH and 0.5 mL of H_2O at 0 °C gave 0.85 g of crude salt, mp 202–203 °C dec, $[\alpha]^{20}_D$ −12.1° (*c* 0.7%, H_2O). Recrystallization of 0.8 g from a mixture of 1 mL of water and 10 mL of 1-butanol gave 0.67 g (65%) of the salt of the desired (−) enantiomer of **4**, mp 203–204 °C dec, $[\alpha]^{20}_D$ −14.8° (*c* 0.9%, H_2O). Anal. ($C_{20}H_{28}N_3O_6P$) C, H, N, P.

Resolution of **4** with (−)-α-methylbenzylamine similarly gave the salt of the (+) enantiomer of **4** (62%), mp 206–208 °C dec, $[\alpha]^{20}_D$ +15.7° (*c* 1%, H_2O). Anal. ($C_{20}H_{28}N_3O_6P$) C, H, N, P.

(−)-(*N*-Glycyl-1-aminoethyl)phosphonic Acid (5b). The (+)-α-methylbenzylamine salt of the (−) enantiomer of **4** (8.4 g, 19 mmol) was converted into the free (−) enantiomer of **4** by cation exchange and hydrogenolyzed in 200 mL of 1:1 MeOH–H_2O with 0.1 mL of AcOH and 0.5 g of 5% Pd/C catalyst. Catalyst and solvent were removed, and the solid residue was triturated with Et_2O and filtered to give **5b**, 3.7 g (97% as monohydrate) (subsequently obtained without hydration, mp 277–280 °C dec, $[\alpha]^{20}_D$ −69.6°), mp 230 °C dec, $[\alpha]^{20}_D$ −61.0° (*c* 1%, H_2O).

Hydrolysis of 5b to 3c. (−)-(*N*-Glycyl-1-aminoethyl)-phosphonic acid (**5b**; 2 g, 11 mmol) was dissolved in 20 mL of 5 N HCl and refluxed for 2.5 h. The solution was evaporated and the residue was dissolved in H_2O and passed down a column of cation-exchange resin. Elution with H_2O left the glycine on the resin and gave five 250-mL fractions containing **3c**. Evaporation, reevaporation with EtOH, and trituration with Et_2O gave 1.2 g of crude **3c**, mp 290–292 °C dec. Recrystallization from a mixture of 7 mL of H_2O and 10 mL of EtOH gave 1.1 g of pure **3c**, mp 294–295 °C dec, $[\alpha]^{20}_D$ −15.8° (*c* 2%, 1 N NaOH).

(+)-(*N*-Glycyl-1-aminoethyl)phosphonic Acid (5a). Similarly to the synthesis of **5b**, the (−)-α-methylbenzylamine salt of the (+) enantiomer of **4** gave (+)-(glycyl-1-aminoethyl)-phosphonic acid (**5a**) (81%), mp 229 °C dec, $[\alpha]^{20}_D$ +65.6° (*c* 1%, H_2O).

(*N*-L-Alanyl-D-1-aminoethyl)phosphonic Acid (9c) (L,D; Table I). The large-scale preparation of **1** (**9d**, L,L) by separation of ester diastereoisomers (Scheme I) had led to the accumulation of large amounts of unwanted isomer **13** (see above).

Compound **13** (179 g, 0.50 mol) was treated with 500 mL of concentrated HCl, SG 1.18, and the mixture was stirred at room temperature overnight. Toluene (200 mL) was then added, and the phases were separated. The aqueous layer was evaporated in vacuo with a bath temperature of 35–40 °C to leave a residue of 202 g of syrup which was dissolved in 400 mL of MeOH and filtered. The filtrate was treated with a solution of 110 mL of propylene oxide in 60 mL of MeOH, dropwise at 30 °C. The mixture was stirred at 0 °C overnight and the solid was filtered and washed with 200 mL of MeOH to give 88 g (90%) of **9c** (L,D), mp 296–297 °C dec, $[\alpha]^{20}_D$ +79.3°; $[\alpha]^{20}_{365}$ +304° (*c* 1%, H_2O); NMR and TLC as for **1** (**9d**, L,L). Recrystallization from 400 mL of H_2O by treatment with 3 g of charcoal and then addition of 1.2 L of EtOH and refrigeration overnight gave 82 g of pure **9c** (L,D), mp 297–298 °C dec, $[\alpha]^{20}_D$ +81.2°; $[\alpha]^{20}_{365}$ 308.5°. Anal. ($C_5H_{11}N_2O_4P$) C, H, N.

Diethyl [L-1-(Benzylideneamino)ethyl]phosphonate (20; Scheme II). Treatment of **3c** with benzyl chloroformate by the literature route for racemic material[9] gave **16** (L), $[\alpha]^{20}_D$ −28.9° (*c* 1%, AcOH), mp 128–130 °C dec. Reaction of **16** (L) with triethyl orthoformate[9] gave 87% of **17** (L), mp 61–62.5 °C, $[\alpha]^{20}_D$ −22.3° (*c* 1%, MeOH). Hydrogenolysis[9] of **17** (L) (24 g, 75 mmol) gave 15.8 g (97%) of **19**, mp 107–107 °C, $[\alpha]^{20}_D$ +2.0° (*c* 1%, H_2O).

Compound **19** (15 g, 70 mmol) was taken up in 120 mL of EtOH and stirred at 0 °C as sodium ethoxide (75 mmol) in 100 mL of EtOH was added. Benzaldehyde (7.5 mL, 75 mmol) was added and the mixture was stirred at room temperature overnight. The mixture was evaporated in vacuo and the residue was partitioned between benzene and water. The benzene extract was dried (Na_2SO_4) and evaporated in vacuo. The residue was distilled with use of a short-path Kugelröhr apparatus at 170–180 °C and 0.1-mmHg pressure to give 15 g (78%) of **20**, n^{21}_D 1.5187, $[\alpha]^{20}_D$ +92.4° (*c* 1%, MeOH). NMR ($CDCl_3$) was as for the racemate.

[L-1-[(*N*-Methyl-L-alanyl)amino]ethyl]phosphonic Acid (23). A mixture of *N*-(benzyloxycarbonyl)-*N*-methyl-L-alanine[17] (2.5 g, 10 mmol) and *N*-hydroxysuccinimide (1.2 g, 10.5 mmol) in 60 mL of dimethoxyethane was treated with dicyclohexylcarbodiimide (2.4 g, 12 mmol) at 0 °C. The precipitated dicyclohexylurea was filtered off and the filtrate was evaporated to give 5.8 g of active ester as an oil. This ester (10 mmol) was coupled with **3c** as for **3** + **7** → **8** (Table I) but in aqueous DMF with Et_3N as base. The product was recrystallized from a mixture of 5 mL of Me_2CO and 25 mL of Et_2O to give 0.7 g of the monobenzylamine salt of [L-1-[[*N*-(benzyloxycarbonyl)-*N*-methyl-L-alanyl]amino]ethyl]phosphonic acid, mp 163–167 °C dec, $[\alpha]^{20}_D$ −31.7° (*c* 0.5%, H_2O). Three further crops gave a total yield of 1.6 g (33%). This salt 1.5 g (3.3 mmol) was converted into the free acid with cation resin and hydrogenolyzed in water over 0.2 g of 10% Pd/C catalyst. The product was recrystallized from a mixture of 8 mL of H_2O and 6 mL of EtOH to give 0.4 g (62%) of [L-1-[(*N*-methyl-L-alanyl)amino]ethyl]phosphonic acid (**23**; Table II). Anal. ($C_6H_{15}N_2O_4P$) C, H, N, P.

[L-1-(L-Alanyl-L-alanylamino)ethyl]phosphonic Acid (36). Coupling of **7a** and **1**, as for **3** + **7** → **8** (Table I) gave the monobenzylamine salt of *N*-carbobenzyloxy **36** in 74% yield, mp 247–250 °C dec, $[\alpha]^{20}_D$ −45.1° (*c* 0.5%, AcOH). Anal. ($C_{23}H_{33}N_4O_7P$) C, H, N, P. Hydrogenolysis, as for **8** → **9** (Table I), gave **36** in 80% yield, mp 283–284 °C dec, $[\alpha]^{20}_D$ −66.8° (*c* 0.5%, H_2O). Anal. ($C_8H_{18}N_3O_5P$) C, H, N, P.

[D-1-(L-Alanyl-L-alanylamino)ethyl]phosphonic Acid (37). Coupling of **7a** and **9c** (L,D), as for **3** and **7** → **8** (Table I), gave the monobenzylamine salt of *N*-carbobenzyloxy **37** in 82% yield, mp 229–231 °C dec, $[\alpha]^{20}_D$ 0.0° (*c* 1%, AcOH). Hydrogenolysis, as for **8** → **9**, gave **37** in 89% yield, mp 290–291 °C dec, $[\alpha]^{20}_D$ +2.9° (*c* 1%, H_2O). Anal. ($C_8H_{18}N_3O_5P$) C, H, N, P.

[L-1-(*N*-Acetyl-L-alanyl-L-alanylamino)ethyl]phosphonic Acid (38). Tripeptide **36** (1.1 g, 4 mmol) was dissolved in 5 mL of 4 N NaOH. Acetic anhydride (1 g, 0.94 mL, 10 mmol) was added at 0 °C. The mixture was stood overnight at room temperature. Water (10 mL) and 15 mL of Et_2O were added. The aqueous layer was diluted with 10 mL of MeOH and passed over cation-exchange resin and evaporated to give 1.1 g (90%), mp 254–255 °C dec. Recrystallization from a mixture of 10 mL of H_2O, 100 mL of EtOH, and 250 mL of Et_2O gave 1.0 g (83%) of **38**, mp 253–255 °C dec, $[\alpha]^{20}_D$ −119° (*c* 1%, H_2O). Anal. ($C_{10}H_{20}N_3O_6P$) C, H, N.

[L-1-(*N*-Acetyl-L-alanyl-L-alanyl-L-alanylamino)ethyl]phosphonic Acid (39). Tetrapeptide (L-Ala)$_3$-L-Ala(P),[2] prepared from **36** above (1.0 g, 3 mmol), was acetylated with acetic anhydride (0.77 g, 0.71 mL, 7.5 mmol) as for **38** above to give 1.0 g of **39**, mp 261–263 °C dec, $[\alpha]^{20}_D$ −140° (*c* 0.6%, H_2O). Anal. ($C_{13}H_{25}N_4O_7P$) C, H, N, P.

[L-1-[(*N*-Methyl-L-alanyl)-L-alanylamino]ethyl]phosphonic Acid (40). Coupling of **1** (4 g, 20 mmol) with the *N*-hydroxysuccinimide ester of *N*-(benzyloxycarbonyl)-*N*-methyl-L-alanine[17] (20 mmol) as for the synthesis of **23** above gave 7.7 g (74%) of the monobenzylamine salt of [L-1-[[*N*-(benzyloxycarbonyl)-*N*-methyl-L-alanyl-L-alanyl]amino]ethyl]phosphonic acid, mp 215–218 °C dec, $[\alpha]^{20}_D$ −58.3° (*c* 0.5%, H_2O). Hydrogenolysis, as above, gave 3.2 g (78%) of [L-1-[(*N*-methyl-L-alanyl-L-alanyl)amino]ethyl]phosphonic acid (**40**), mp 289–291 °C dec, $[\alpha]^{20}_D$ −80.1° (*c* 0.5%, H_2O).

[L-1-[(*N*-Methyl-L-alanyl-L-alanyl-L-alanyl)amino]ethyl]phosphonic Acid (41). Phosphonotripeptide **36** (2.8 g, 10.5 mmol) was reacted with the *N*-hydroxysuccinimide ester of *N*-(benzyloxycarbonyl)-*N*-methyl-L-alanine[17] (10.5 mmol) as for

the preparation of **23** above. Workup as above gave 2.6 g (50%) of the free [L-1-[[*N*-(benzyloxycarbonyl)-*N*-methyl-L-alanyl-L-alanyl-L-alanyl]amino]ethyl]phosphonic acid, mp 225–226 °C dec, $[\alpha]^{20}_D$ -99.3° (*c* 0.5%, H_2O). Hydrogenolysis of this material (2.4 g, 5 mmol) over 0.25 g of 10% Pd/C catalyst gave, after crystallization from a mixture of 30 mL of H_2O and 30 mL of EtOH, 1.3 g (76%), mp 314–316 °C dec. Recrystallization from a mixture of 50 mL of H_2O and 25 mL of EtOH gave 0.22 g of (**41**), mp 318–320 °C dec, $[\alpha]^{20}_D$ -121° (*c* 0.5%, H_2O).

[L-1-[(L-Alanyl-*N*-methyl-L-alanyl)amino]ethyl]phosphonic Acid (42). *N*-(Benzyloxycarbonyl)-L-alanine[17] (18 g, 80 mmol) was stirred in 600 mL of petroleum ether with *N*-ethylmorpholine (9.2 g, 80 mmol) and cooled to -5 °C. Isobutyl chloroformate (11 g, 80 mmol) was added to the mixture which was maintained at -5 °C for 0.5 h. A solution of **23** (1.7 g, 8 mmol), prepared as above, in 8 mL of H_2O containing *N*-ethylmorpholine (1.8 g, 16 mmol) was added at -5 °C. The mixture was stirred for 1 h at -5 °C and then overnight at room temperature. Workup as for **4** followed by catalytic hydrogenolysis and recrystallization from a mixture of 7 mL of MeOH and 7 mL of Me_2CO gave 0.6 g (26%) of **42**, mp ca. 175 °C dec, $[\alpha]^{20}_D$ -62.4° (*c* 1%, H_2O). Anal. ($C_9H_{20}N_3O_5P$) C, H, N. P. TLC showed a double spot at R_f 0.4–0.6 and NMR suggested that this product was probably a mixture of cis and trans isomers (1:9 ratio) at the amide *N*-methyl bond.

[L-1-[(L-Alanyl-*N*-methyl-L-alanyl-L-alanyl)amino]ethyl]phosphonic Acid (43). Coupling of **40** (1.7 g, 6 mmol), as above, with *N*-(benzyloxycarbonyl)-L-alanine (16 g, 72 mmol) by the mixed anhydride method, as for **42** above, followed by hydrogenolysis gave 0.5 g of solid. Two recrystallizations from 14 mL and then 3 mL of 1:1 MeOH–EtOAc gave 0.17 g (8%) of **43**, mp 168 °C dec, $[\alpha]^{20}_D$ -100° (*c* 0.5%, H_2O). Anal. ($C_{12}H_{25}N_4O_6P$) C, H, N, P. TLC and NMR again suggested that this material was a mixture of cis and trans isomers in 1:9 ratio at the amide *N*-methyl bond.

[L-1-(L-Norvalylamino)ethyl]phosphonic Acid (45). Treatment of L-norvaline (5.0 g, 42.5 mmol) with benzyl chloroformate (10 g, 60 mmol) and sodium hydroxide, as for the preparation of *N*-(benzyloxycarbonyl)glycine,[32] and crystallization from 10 mL of Et_2O and 20 mL of petroleum ether, bp 60–80 °C, gave 8.2 g (77%) of *N*-(benzyloxycarbonyl)-L-norvaline, mp 85–87 °C, $[\alpha]^{20}_D$ -9.9°, $[\alpha]^{20}_{365}$ -26.5° (*c* 1%, EtOH). This acid (8.1 g, 32 mmol) and *N*-hydroxysuccinimide (3.7 g, 32 mmol) in 75 mL of dimethoxyethane with dicyclohexylcarbodiimide (7.2 g, 35 mmol), as for **23**, gave, on trituration with EtOH, 9.8 g of *N*-(benzyloxycarbonyl)-L-norvaline *N*-hydroxysuccinimide ester, mp 95–97 °C, $[\alpha]^{20}_D$ -35.1° (*c* 1%, EtOH). The active ester (9.8 g, 28 mmol) was coupled at 0 °C with **3c** (4.4 g, 35 mmol) in 120 mL of 1:2 aqueous DMF with Et_3N (7.1 g, 70 mmol) as base and stirred overnight at room temperature. The mixture was evaporated in vacuo. The residue was dissolved in H_2O–MeOH (1:1) and passed over cation-exchange resin (RSO_3H). The acid eluate was titrated to pH 4.5 with benzylamine to give 9.6 g (73%) of the monobenzylamine salt of [L-1-[(*N*-benzyloxycarbonyl-L-norvalyl)amino]ethyl]phosphonic acid, mp 225–230 °C dec, $[\alpha]^{20}_D$ -29.9°, $[\alpha]^{20}_{365}$ -98.8° (*c* 1%, AcOH). This salt (9.0 g, 19 mmol) was stirred at room temperature for 6 h with a mixture of 20 mL of 45% HBr in AcOH and 8 mL of AcOH. Workup as for alafosfalin (**1**) (method a) and recrystallization from a mixture of 80 mL of H_2O and 160 mL of EtOH gave 3.5 g (82%) of [L-1-(L-norvalylamino)ethyl]phosphonic acid (**45**), mp 260–262 °C, $[\alpha]^{20}_D$ -19.5°, $[\alpha]^{20}_{365}$ -75.3° (*c* 1%, H_2O).[5] Anal. ($C_7H_{17}N_2O_4P$) C, H, N, P.

[L-1-[(Sarcosyl-L-norvalyl-L-norvalyl)amino]ethyl]phosphonic Acid (46). To a mixture of dipeptide **45** (3.4 g, 15 mmol), 30 mL of H_2O, Et_3N (3 g, 30 mmol), and DMF (30 mL) at 0 °C was added *N*-(benzyloxycarbonyl)-L-norvaline *N*-hydroxysuccinimide ester (7.8 g, 22.5 mmol) (see above) in 30 mL of DMF. The mixture was stirred overnight then worked up as for **45** above to give 7.8 g (92%) of salt, mp 243–245 °C dec. Recrystallization of 0.5 g from 200 mL of H_2O gave 0.18 g of pure monobenzylamine salt of [L-1-[[*N*-(benzyloxycarbonyl)-L-norvalyl-L-norvalyl]amino]ethyl]phosphonic acid, mp 247–249 °C dec, $[\alpha]^{20}_D$ -35.7° dec, $[\alpha]^{20}_{365}$ -125° (*c* 0.5%, AcOH). Anal. ($C_{27}H_{41}N_4O_7P$) C, H, N.

This salt (7.8 g, 14 mmol) was stirred with a mixture of 15 mL of 45% HBr in AcOH and 5 mL of AcOH for 5 h at room temperature. Workup as for **45** gave 4 g of product, mp 260–265 °C dec. This was stirred with 250 mL of water and then filtered to give 3.7 g (83%) of [L-1-[(L-norvalyl-L-norvalyl)amino]ethyl]-phosphonic acid, mp 273–275 °C dec, $[\alpha]^{20}_D$ -36.0°, $[\alpha]^{20}_{365}$ -153° (*c* 0.5%, 0.1 N NaOH).

To a mixtue of *N*-(benzyloxycarbonyl)sarcosine[33] (60 g, 0.27 mol) and *N*-hydroxysuccinimide (31 g, 0.27 mol) in 400 mL of dimethoxyethane at 0 °C was added dicyclohexylcarbodiimide (62 g, 0.30 mol). The mixture was stood at 0 °C overnight. The solid byproduct was filtered off and the filtrate was evaporated. The residual oil was taken up in 400 mL of DMF to give a stock solution of *N*-(benzyloxycarbonyl)sarcosine *N*-hydroxysuccinimide ester containing 0.5 mmol/mL, assuming a 75% yield. [This solution was also used to prepare other sarcosyl tripeptides[22] (e.g., Table V).]

To a mixture of L-norvalyl tripeptide (3.4 g, 10.5 mmol), prepared above, 30 mL of H_2O, 30 mL of DMF, and Et_3N (2.1 g, 21 mmol) was added at 0 °C the active ester (31.5 mL of DMF solution, 16 mmol), prepared as above. The mixture was stirred overnight at room temperature. A little solid was filtered off and the filtrate was evaporated in vacuo. The residue was stirred with 200 mL of H_2O and 2 N HCl was added to pH 1 and stirring was continued for several hours. The gelatinous precipitate was filtered off to give 5.9 g of solid, mp 210–214 °C dec. This solid was stirred with 75 mL of Me_2CO and filtered to give 5.0 g (90%) of [L-1-[(*N*-benzyloxycarbonyl)sarcosyl-L-norvalyl-L-norvalyl)amino]-ethyl]phosphonic acid, mp 217–219 °C dec, $[\alpha]^{20}_D$ -30.2°, $[\alpha]^{20}_{365}$ -113° (*c* 0.5%, DMSO).

This material (4.6 g, 8.8 mmol) was stirred with a mixture of 15 mL of 45% HBr in AcOH and 5 mL of AcOH for 5 h at room temperature. Workup as for **45** above gave 3.25 g (94%) of product, mp 272–275 °C (dec. Recrystallization from a mixture of 500 mL of H_2O and 1.5 L of EtOH gave 2.85 g (82%) of **46**,[5,22] mp 275–278 °C dec, $[\alpha]^{20}_D$ -83.5°, $[\alpha]^{20}_{365}$ -306° (*c* 0.4%, H_2O).

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Registry No. deuterio-**1**, 98820-81-6; **3a**, 16606-65-8; deuterio-**3a**, 98820-79-2; **3b**, 66068-76-6; **3c**, 60687-36-7; deuterio-**3c**, 98857-07-9; **4** (monobenzylamine salt), 70858-91-2; (-)-**4**·(mono-(+)-α-methylbenzylamine salt), 98820-83-8; (+)-**4**·(mono-(-)-α-methylbenzylamine salt), 98820-84-9; **5a**, 66023-95-8; **5b**, 60668-42-0; **7a**, 3401-36-3; **7b**, 27167-53-9; **8a**, 98820-76-9; **8a** (monobenzylamine salt), 98820-77-0; **8b**, 98820-75-8; **8b** (monobenzylamine salt), 98820-78-1; **8c**, 60668-66-8; **8c** (monobenzylamine salt), 60668-67-9; **8d**, 60668-26-0; deuterio-**8d**, 98820-82-7; **8d** (monobenzylamine salt), 60668-27-1; **9a**, 54772-83-7; **9b**, 98857-06-8; **9c**, 66023-94-7; **9d**, 60668-24-8; **12**, 79646-31-4; **13**, 60668-36-2; **14**, 67739-69-9; **15** (R = CH_2F), 98821-02-4; **15** (R = $(CH_2)_3NHC(NH)NHNO_2$), 66449-72-7; **15** (R = $(CH_2)_4NHC(NH)NH_2$), 71447-97-7; **15** (R = CH_2SCH_3), 82638-13-9; **15** (R = $CH_2SCH_2CH_2CH_3$), 98821-03-5; **15** (R = $CH_2CH_2S(O)CH_3$), 98821-04-6; **15** (R = $CH_2CH_2S(O)_2CH_3$), 98857-11-5; **15** (R = $CH_2C{=}CHCH{=}CHS$), 98857-12-6; L-**16**, 60687-34-5; L-**17**, 98857-08-0; **19**, 98820-85-0; **20**, 98820-80-5; **23**, 98857-09-1; **24**, 98838-19-8; **25**, 66469-49-6; **26**, 98820-93-0; **27**, 98820-94-1; **28a**, 98820-95-2; **28b**, 98820-96-3; **29a**, 98820-97-4; **29b**, 98820-98-5; **30**, 74892-96-9; **31**, 60668-23-7; **32**, 82638-08-2; **33**, 98820-99-6; **34**, 98821-00-2; **35**, 98821-01-3; **36**, 65621-17-2; *N*-carbobenzyloxy-**36** (monobenzylamine salt), 70878-88-5; **37**, 60778-50-9; *N*-carbobenzyloxy-**37** (monobenzylamine salt), 98857-10-4; **38**, 67008-87-1; **39**, 66381-27-9; **40**, 98820-88-3; **41**, 71922-18-4; **42**, 98820-91-8; **43**,

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Quantitative Structure–Activity Relationship of the Mutagenicity of Substituted *N*-Nitroso-*N*-benzylmethylamines: Possible Implications for Carcinogenicity†

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The relative mutagenicities of substituted *N*-nitroso-*N*-benzylmethylamines have been reexamined from a quantitative structure–activity relationship point of view. Most of the compounds were mutagenic toward *Salmonella typhimurium* TA 1535 with Aroclor-induced male hamster liver S9 activation. The dose–response data were subjected to a multiple linear regression equation calculated in a stepwise manner, which found that the differences in mutagenicities could be explained primarily by differences in the three-bond path molecular connectivity index, with smaller contributions from σ and π. Moreover, a polynomial regression analysis showed that the maximum mutagenicity could be explained by an optimal amount of electron withdrawal by the substituent which would cause a weakening, or activation, of the methylene C–H bond. The possible relevance of these observations to carcinogenesis is discussed.

In a recent study[1] we showed that the mutagenicity of para-substituted *N*-nitroso-*N*-benzylmethylamines toward *Salmonella typhimurium* TA 1535 varied as the substituent was changed. It appeared that there was an optimal amount of electron withdrawal necessary for maximum mutagenicity, but the number of data points available was not great enough to allow us to carry out definitive calculations or to reach any firm conclusions concerning the physicochemical parameters responsible for the mutagenic effects.

We have now prepared additional related compounds and have tested the entire set for mutagenicity against the TA 1535 strain. Multiple regression analyses of the data successfully showed several relationships between the mutagenicity data and physicochemical parameters. These observations may also be relevant to the carcinogenicity of nitrosamines.

Results

We knew from our previous study[1] that strain TA 1535 was the most sensitive of the Ames strains toward these compounds and thus this was the only strain we employed in this study. We also knew that activation was appreciably more effective with the S9 fraction from Aroclor-stimulated male hamster liver than from rat liver for these nitrosamines, as it is for most other nitrosamines.[2] The *p*-methyl, *p*-carboxyl, and the *p*-carboxymethyl derivatives were not mutagenic at any dose tested, and the *p*-methoxy derivative was not mutagenic in this experiment although it has shown moderate mutagenicity in previous trials (ref 1 and G. M. Singer and A. W. Andrews, unpublished data). These inactive compounds were not included in derivation of the quantitative structure–activity relationship (QSAR) equations. The detailed mutagenicity data from the dose–response assays are shown in Table I. As the index for mutagenic potency we chose the molar concentration of the nitrosamine which induced 50 revertants/plate. This point was on the linear portion of the dose–response curves for all of the active compounds (Figure 1). This index was chosen in preference to the initial slope of the dose–response curves because the length of the linear portion of the curve was different for each compound. For the more potent compounds, the linear portion was often only three points.

We analyzed the mutagenicity data using both a multiple linear regression model and also a polynomial regression model. The physicochemical parameters submitted to the linear regression analysis for each compound were the Hammett σ constant,[3] Hansch's π factor [log (P_X/P_H)], molar refractivity,[4] and two different molecular connectivity indices,[5] $^1\chi^v$ and $^3\chi_P^v$ (Table II). The π

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