Total Synthesis and Enzyme-Substrate Interaction of D-, DL-, and L-Phosphinotricine, 'Bialaphos' (SF-1293) and Its Cyclic Analogues

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DL-Phosphonotricine (**3**) and its cyclic analogue (**4**) have been synthesized using the four-component isocyanide condensation of Ugi and Ugi-analogous three-component condensation, respectively. High selectivity of the enzyme–substrate interaction was established with the enzymes α-chymotrypsin, phosphodiesterase I, and alkaline mesintericopeptidase, as well as by separation of the racemic mixture to optical antipodes by the α-chymotripsin. The tripeptide 'bialaphos' (**11**) and its D-antipode (**11a**) have been synthesized by the method of activated esters, and the cyclic analogue (**15**) by the DCC method. It was found that the phospholane L-(**5**) and the tripeptide (**18**) exhibit anti-tumour activity.

γ-[Hydroxy(methyl)phosphinoyl]-L-α-aminobutyryl-L-alanyl-L-alanine (**11**), generally called bialaphos (SF-1293), is a tripeptide antibiotic, produced from *Streptomyces viridochromogenes* strains[1] and exhibits well pronounced herbicidal and fungicidal activity.[2,3]

The P methyl ester of DL-phosphinotricyl-DL-alanyl-DL-alanine was synthesized *via* a multi-step sequence[4] in a yield of *ca.* 30%. Enzymatic degradation of the natural tripeptide with *E. coli* protease was shown[5] to give L-phosphinotricine and L-alanine.

2-Amino-4-[hydroxy(methyl)phosphinoyl]-L-butyric acid [L-(**2**)], known as L-phosphinotricine (a name given by Bayer *et al.*[1]), was isolated from *Streptomyces hydroscopic* strains[6] and has recently been synthesized[7] by Michael addition of vinylphosphorus compounds to the chiral glycine Schiff base. There is another method,[8] published recently, for the synthesis of D- and L-phosphinotricine using Schölkopf's method.

The racemic mixture of phosphinotricine, obtained synthetically,[9,10] is widely used as a fungicide and herbicide.[11]

In this work, a suitable method is proposed for the synthesis of D-, DL-, and L-phosphinotricine. The free tripeptide bialaphos has been synthesized for the first time by use of enzymes which provide selective hydrolyses of phosphonates in the presence of ethoxycarbonyl and peptide bonds. The kinetics of the enzyme–substrate interaction involving esters of some other organophosphorus acids with phosphodiesterase-like enzymes has been studied: 4-nitrophenyl benzenephosphonate with calf intestinal alkaline phosphatase[12] and mononitrophenyl and 2-naphthyl methane-, benzene-, and chloromethanephosphonate with intestinal 5′-nucleotide phosphodiesterase.[13] The enzyme alkaline mesintericopeptidase (EC 3.4.4) has been used for the selective hydrolysis[14] of the ethoxycarbonyl group of unusual substrates [*e.g.* H-Cys(O_2NH_2)-OEt] to the corresponding free acids. Up to now this enzyme has not been employed with derivatives of amino acids and peptides, containing organophosphorus acid residues.

Results and Discussion

Synthesis of D-, DL-, *and* L-*Phosphinotricine and Its Cyclic Analogues.*—A key approach to the synthesis of phosphinotricine was a four-component isocyanide (RNC) condensation following the method of Ugi.[15] Optically active products were obtained by enzyme hydrolysis and separation of the racemic mixture with α-chymotrypsin.

The condensation was carried out by successive addition of the aldehyde (**1**)[16] and cyclohexyl isocyanide to a solution of ammonium formate in 80% aqueous methanol. Mineral acid hydrolysis of the reaction products gave the hydrochloride of DL-phosphinotricine (**3**) in a yield of 60—65% [based on the amount of the aldehyde (**1**) used].

Using the recently synthesized[17] aldehyde (**2**) as a carbonyl component (without a carboxy group) an unexpected cyclization occurred to give the 1,2-azaphospholidine (**4**) [yield *ca.* 70% based on the amount of the aldehyde (**2**) used]. Since the azaphospholidine (**4**) may be considered as a phosphino analogue of pyroglutamic acid it is named as pyrophosphinotricine. Upon mineral acid hydrolysis it gave a satisfactory yield of the DL-phosphinotricine (**3**). Despite the yield of phosphinotricine (**3**) being in both cases almost the same, the second procedure with the azaphospholidine (**4**) is much to be preferred.

D- and L-Phosphinotricine (**3**) were obtained by α-chymotrypsin hydrolyses of two different substrates.

With the 1,2-azaphospholidine (**4**) as substrate, the hydrolysis occurred only with the L-form of the racemic mixture, yielding, almost quantitatively, unchanged D-(**4**) and the free acid L-(**5**).

Similarly, D-phosphinotricine, D-(**3**), and L-phosphinotricine, L-(**3**), were obtained by mineral acid hydrolysis of the azaphospholidine D-(**4**) and L-(**5**), respectively.

With the diethyl ester of DL-phosphinotricine (**6**) as substrate, readily obtained as a hydrogen chloride salt by sequential treatment of DL-phosphinotricine (**3**) with phosphorus pentachloride and ethanol, enzyme-catalyzed hydrolysis with α-chymotrypsin gone, almost quantitatively, D-(**6**) and the phosphino ester of L-phosphinotricine, L-(**7**).

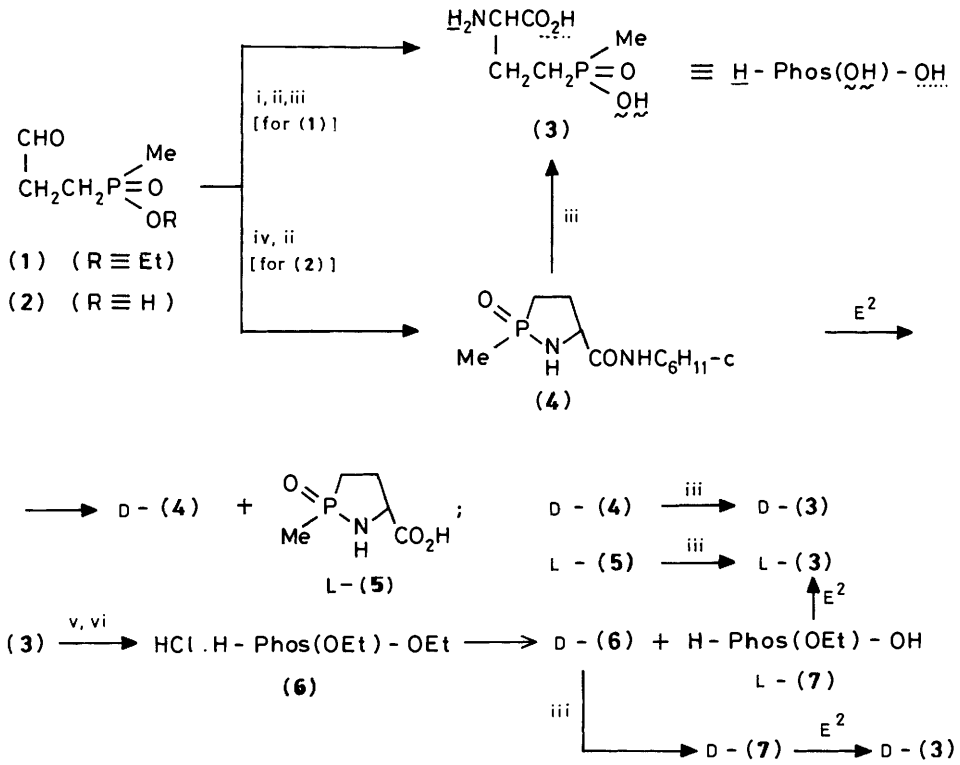
It was found that the enzyme phosphodiesterase I, widely used to hydrolyse esters of phosphorus acids, could successfully be applied to the esters of phosphinic acids[18] as well. By this approach with the phosphino ester L-(**7**) as a substrate, the L-phosphinotricine L-(**3**) was prepared in practically quantitative yield. It is worth noting that the ethoxycarbonyl and peptide groups were unaffected and that the ability of the compound to undergo enzyme hydrolysis with phosphodiesterase I was retained in all the procedures described here.

'Mild' mineral acid hydrolysis of the ethoxycarbonyl group of D-(**8**), with retention of the phosphino ester group, gave the phosphino ester of phosphinotricine D-(**7**) (yield of *ca.* 85%).

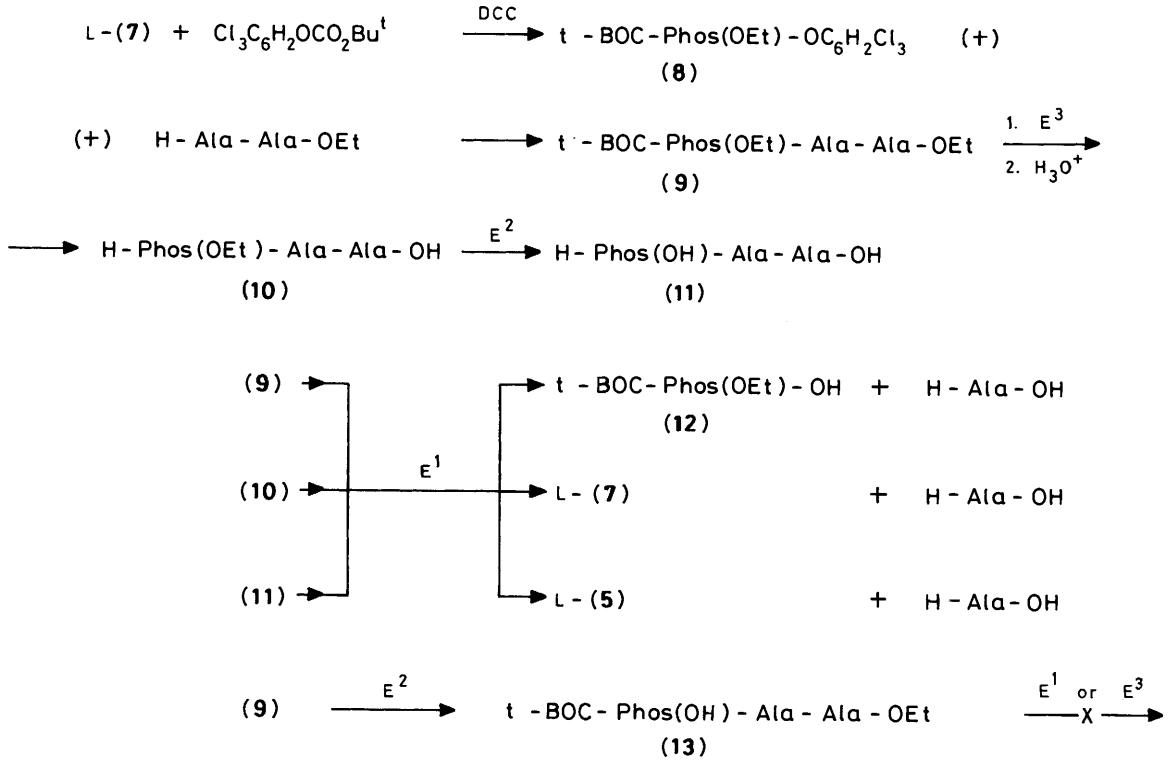
Enzyme-catalyzed hydrolysis of the substrate D-(**7**) with phosphodiesterase I also provided a practically quantitative yield of the D-phosphinotricine D-(**3**), which was identical with that obtained by mineral acid hydrolysis of azaphospholidine D-(**4**).

Synthesis of Bialaphos and Its Analogues.—To synthesize the natural tripeptide bialaphos (SF-1293) the activated ester method was employed.

An analogous method to that developed in the original paper[19] was applied to the reaction system of t-butyl 2,4,5-



Scheme 1. $E^1 \equiv$ α-chymotrypsin; $E^2 \equiv$ phosphodiesterase I. *Reagents:* i, HCO_2NH_4; ii, c-$C_6H_{11}NC$; iii, H_3O^+; iv, NH_3; v, PCl_5; iv, EtOH



Scheme 2. $E^3 \equiv$ alkaline mesintericopeptidase

trichlorophenyl carbonate and L-(7) to yield L-phosphonitricine (**8**), with completely protected functional groups. The reaction of (**8**) with L-alanyl-L-alaninoethyl ester to give the protected tripeptide (**9**) was carried out by the method introduced in references 19 and 20.

Liberation of the C-end of the tripeptide (**9**) was achieved through enzyme–substrate interaction with alkaline mesintericopeptidase. Unlike the above processes involving enzymes, here the hydrolysis must be strictly controlled, in order to avoid the enzyme hydrolysis, albeit slowly, of the peptide bonds as

Table 1. Physical data for the synthesized compounds (**3**)—(**19**)

Compound (Formula)	Yield (%)	M.p. (°C) (decomp.)	$[\alpha]_D^{20}$	Found (%) (Required) C	H	N	R_F	*M* Found (Required)
(**3**)	63	(205—207)	DL-form	27.8	6.0	6.65	0.40	179
($C_5H_{12}NO_4P$·HCl)		202—204[9]		(27.6	6.0	6.4)		(181.1)
D-(**3**)	82 D-(**4**)	(≃200)	−13.3	27.6	6.1	6.6	0.40	183
($C_5H_{12}NO_4P$·HCl)	100 D-(**7**)		−12.4[7]	(27.6	6.0	6.4)		(180.1)
L-(**3**)	95 L-(**5**)	(200—202)	+14.1	27.8	5.5	6.25	0.40	177
($C_5H_{12}NO_4P$·HCl)	100 L-(**7**)	197—199[9]	+13.4[7] +15.9[9]	(27.6	6.0	6.4)		(180.1)
(**4**)	73	(218—220)	DL-form	54.4	8.4	11.4	0.43	244
($C_{11}H_{21}N_2O_2P$)				(54.1	8.6	11.4)		(244.3)
D-(**4**)	≃100	(209—211)	−62.8	54.3	8.8	11.2	0.43	248
($C_{11}H_{21}N_2O_2P$)				(54.1	8.6	11.4)		(244.3)
L-(**5**)	≃100	(186—190)	+69.7	37.0	6.0	8.6	0.63	163
($C_5H_{10}NO_3P$)				(36.8	6.2	8.6)		(163.1)
(**6**)	72	(155—158)	DL-form	39.8	7.6	4.9	0.49	269
($C_9H_{20}NO_4P$·HCl)				(39.5	7.7	5.1)		(273.2)
D-(**6**)	≃100	(163—164)	−18.9	39.6	7.9	5.2	0.49	274
($C_9H_{20}NO_4P$·HCl)				(39.5	7.7	5.1)		(273.2)
D-(**7**)	85	180—183	−17.6	34.5	7.2	5.8	0.62	207
($C_7H_{16}NO_4P$·HCl)				(34.2	7.0	5.7)		(209.2)
L-(**7**)	≃100	176—179	+14.3	39.9	8.0	6.7	0.62	205
($C_7H_{16}NO_4P$·HCl)				(40.2	7.7	6.7)		(209.2)
(**8**)	88	110—113	+59.3	44.7	5.0	3.2	0.52	482
($C_{18}H_{25}Cl_3NO_6P$)				(44.3	5.2	2.9)		(488.7)
				Cl: 22.0 (21.8)				
(**8a**)	86	108—110	−60.1	44.5	5.2	3.3	0.52	484
($C_{18}H_{25}Cl_3NO_6P$)				(44.3	5.2	2.9)		(488.7)
				Cl: 22.2 (21.8)				
(**9**)	84	143—147	−33.6	50.3	7.7	9.0	0.62	482
($C_{20}H_{38}N_3O_8P$)				(50.1	8.0	8.8)		(479.5)
(**9a**)	86	140—142	−56.8	49.9	8.1	8.85	0.62	475
($C_{20}H_{38}N_3O_8P$)				(50.1	8.0	8.8)		(479.5)
(**10**)	85	Amorphous mass	−30.2	44.4	7.6	12.0	0.40	351
($C_{13}H_{26}N_3O_6P$)				(44.4	7.5	12.0)		(351.3)
(**10a**)	87	Amorphous mass	−60.8	44.7	7.2	12.1	0.40	354
($C_{13}H_{26}N_3O_6P$)				(44.4	7.5	12.0)		(354.3)
(**11**)	≃100	161—163	−32.3	41.0	6.5	12.9	0.32	327
($C_{11}H_{22}N_3O_6P$)		159—161[a]	−34.0[20]	(40.9	6.9	13.0)		(323.3)
(**11a**)	≃100	(170—173)	−53.2	41.1	6.6	13.2	0.32	320
($C_{11}H_{22}N_3O_6P$)				(40.9	6.9	13.0)		(323.3)
(**12**)	90	Amorphous mass	+68.7	46.4	8.0	4.4	0.46	308
($C_{12}H_{24}NO_6P$)				(46.6	7.8	4.5)		(309.3)
(**13**)	≃100	Amorphous mass	−36.2	47.6	7.5	9.2	0.83	456
($C_{18}H_{34}N_3O_8P$)				(47.9	7.6	9.3)		(451.5)
(**13a**)	≃100	Amorphous mass	−60.4	48.2	7.4	9.0	0.83	456
($C_{18}H_{34}N_3O_8P$)				(47.9	7.6	9.3)		(451.5)
(**14**)	93	Amorphous mass	−55.3	47.2	7.7	7.2	0.44	384
($C_{15}H_{29}N_2O_2P$)				(47.4	7.7	7.4)		(384.4)
(**15**)	95	(173—176)	−58.3	42.6	7.8	9.9	0.53	283
($C_{10}H_{21}N_2O_5P$)				(42.9	7.55	10.0)		(280.2)
(**16**)	93	(196—200)	−61.2	38.2	6.5	11.0	0.41	254
($C_8H_{17}N_2O_5P$)				(38.1	6.6	11.1)		(252.2)
(**17**)	73	(173—176)	−18.3	46.95	7.0	12.7	0.42	330
($C_{13}H_{20}N_3O_5P$)				(46.8	7.3	12.6)		(333.3)
(**18**)	92	(220)	−22.4	43.4	6.3	13.8	0.44	302
($C_{11}H_{20}N_3O_5P$)				(43.3	6.6	13.8)		(305.3)
(**19**)	94	(200)	−14.3	40.9	6.8	12.1	0.37	236
($C_8H_{15}N_2O_4P$)				(41.0	6.5	12.0)		(234.2)

[a] T. Niida, S. Inone, T. Tsurueka, T. Shomura, Y. Kondo, T. Sekizawa, T. Watanabe, and H. Igarash, BRD 2 236 599/1973.

well. Liberation of the N-end of the tripeptide (**9**), which leads to the tripeptide (**10**), was achieved by the usual peptide synthesis procedure without isolating the intermediate product.

Unfortunately, mineral acid hydrolysis of the ethoxymethylphosphinoyl groups of the tripeptides (**9**) and (**10**) was found to hydrolyse the ethoxycarbonyl and peptide groups too. That is why for the synthesis of the free tripeptide 'bialaphos' a highly selective enzyme-catalyzed hydrolysis needs to be employed. The phosphodiesterase I was used to give the free tripeptide bialaphos (**11**) from (**10**) in practically quantitative yield.

Table 2. Spectral data for compounds (**3**)—(**19**)

Compd.	$\nu_{max.}$(KBr)/cm^{-1}	N.m.r. δ (250 MHz; TMS) (solvent)	Mass spectra Found M^+ (formula, required M^+); m/z (M^+, %)	Content of the enzyme α-chymotrypsin hydrolysate
(**3**) D-(**3**) L-(**3**)	1 740 (CO), 1 510 (NH_3^+), 1 300 (P–CH_3), 1 250 (P=O)	(D_2O) 1.83 (3 H, d, *J* 14 Hz, PCH_3), 2.1—2.8 (4 H, m, PCH_2CH_2), 4.45 (1 H, t, *J* 6 Hz, $CHCH_2$)	181.1529 ($C_5H_{12}NO_4P$, 181.1513) 181 (16)	
(**4**) D-(**4**)	1 645 (CONH), 1 385 (P–N), 1 300 (P–CH_3), 1 200—1 150 (P=O)	([$^2H_6$]-DMSO) 0.7—2.8 (17 H, m, 7 × CH_2, PCH_3), 4.11 (1 H, m, CHN), 4.52 (1 H, t, *J* 6 Hz, CHCO), 5.2—5.4 (1 H, br, NH)	244.3133 ($C_{11}H_{21}N_2O_2P$, 244.3142); 244 (18)	D-(**4**) and L-(**5**) [from (**4**)]
L-(**5**)	3 320—2 860 (CO_2H), 1 385 (P–N), 1 320 (P–CH_3), 1 255 (P=O)	(D_2O) 1.71 (3 H, d, *J* 17 Hz, PCH_3), 2.2—2.7 (4 H, m, PCH_2CH_2), 4.48 (1 H, t, *J* 6 Hz, CH_2CHCO)	163.1333 ($C_5H_{10}NO_3P$, 163.13235); 163 (26)	
(**6**) D-(**6**)	3 400—3 300 (NH_2), 1 740 (CO), 1 320 (P–CH_3), 1 250 (P=O), 1 120—980 (P–O–C)	($CDCl_3$) 1.2—1.8 (12 H, m, 4 × CH_3), 2.2—2.7 (4 H, m, PCH_2CH_2), 3.1—3.6 (2 H, br, NH_2), 3.8—4.3 (5 H, m, 2 × OCH_2CH_3, $CHCH_2$)	237.2756 ($C_9H_{20}NO_4P$, 237.27425); 237 (14)	
D-(**7**) L-(**7**)	3 600—2 860 ($CO_2NH_3^+$), 1 745 (CO), 1 330 (P–CH_3), 1 250 (P=O), 1 110—975 (P–O–C)	(D_2O) 1.29 (3 H, t, *J* 6 Hz, OCH_2CH_3), 1.53 (3 H, d, *J* 16 Hz, PCH_3), 2.2—2.7 (4 H, m, PCH_2CH_2), 4.12 (1 H, t, *J* 6 Hz, $CHCH_2$), 4.25 (2 H, q, *J* 5.5 Hz, OCH_2CH_3)	209, 1 841 ($C_7H_{16}NO_4P$, 209.1837); 209 (32)	D-(**3**) [from D-(**7**)]; L-(**3**) [from D-(**3**)] (with phosphodiesterase I)
(**8**) (**8a**)	1 740—1 640 (CO), 1 320 (P–CH_3), 1 250 (P=O), 1 110—960 (P–O–C), 830 and 780 (Ar)	($CDCl_3$)1.1—1.8 (15 H, m, 5 × CH_3), 2.2—2.7 (4 H, m, PCH_2CH_2), 4.05 (1 H, t, *J* 6 Hz, $CHCH_2$), 4.56 (2 H, q, *J* 5 Hz, OCH_2CH_3), 7.29 and 7.50 (2 H, s, Ar)	488.73605 ($C_{18}H_{25}Cl_3NO_6P$, 488.7355); 488 (8)	
(**9**) (**9a**)	1 775 (CO-ester), 1 730 (CO urethane), 1 655 (CO I), 1 545 (CO II), 1 305 (P–CH_3), 1 250 (P=O), 1 110-940 (P–O–C)	($CDCl_3$) 1.0—1.8 (24 H, m, 8 × CH_3), 2.2—2.7 (4 H, m, PCH_2CH_2), 4.03 (1 H, t, *J* 7 Hz, $CHCH_2$), 4.1—4.4 (6 H, m, 2 × $CHCH_3$, 2 × OCH_2CH_3), 5.2—5.6 (3 H, br, 3 × NH)	479, 5 136 ($C_{20}H_{38}N_3O_8P$, 479.51496); 479 (0.16)	(**12**) and alanine from (**9**); (**14**) and alanine from (**9a**)
(**10**) (**10a**)	3 500—2 800 (CO_2H, NH_2), 1 650 (CO I), 1 545 (CO II), 1 300 (P–CH_3), 1 250 (P=O), 1 110—935 (P–O–C)	([$^2H_6$]-DMSO) 1.3—1.8 (12 H, m, 4 × CH_3), 2.2—2.7 (4 H, m, PCH_2CH_2), 3.1—3.6 (2 H, br, NH_2), 4.06 (1 H, t, *J* 6 Hz, $CHCH_2$), 4.16 (1 H, q, $CHCH_3$), 4.2—4.4 (3 H, m, OCH_2CH_3, $CHCH_2$), 5.6 (2 H, br, 2 × NH), 10.6—11.6 (1 H, br, CO_2H)	351.3418 ($C_{13}H_{26}N_3O_6P$, 351.34247); 351 (0.25)	L-(**7**) and alanine from (**10**); (**15**) and alanine from (**11a**)
(**11**) (**11a**)	3 500—2 800 (CO_2H, NH_2), 1 650 (CO I), 1 540 (CO II), 1 300 (P–CH_3), 1 245 (P=O), [1 650 (CO I), 1 540 (CO II)][a]	(D_2O) 1.29 (3 H, d, *J* 6 Hz, $CHCH_3$), 1.35 (3 H, d, *J* 14 Hz, PCH_3), 1.82 (2 H, m, PCH_2CH_2), 2.21 (2 H, m, PCH_2CH_2), 3.65 (1 H, q, $CHCH_3$), 4.12 (1 H, t, *J* 7 Hz, $CHCH_2$), 4.35 (1 H, q, $CHCH_3$)	323.2874 ($C_{11}H_{22}N_3O_6P$, 323.28839); 323 (0.42)	L(**3**) and alanine from (**11**); (**16**) and alanine from (**11a**)
(**12**)	3 500—2 850 (CO_2H), 1 750—1 770 (CO), 1 730 (CO-urethane), 1 330 (P–CH_3), 1 255 (P=O), 1 110—975 (P–O–C)	([$^2H_6$]-DMSO) 1.0—1.8 (15 H, m, 5 × CH_3), 2.2—2.7 (4 H, m, PCH_2CH_2), 4.08 (1 H, t, *J* 6 Hz, $CHCH_2$), 4.20 (2 H, q, OCH_2CH_3), 5.20 (1 H, br, NH), 11.0—11.6 (1 H, br, CO_2H)	309.3011 ($C_{12}H_{24}NO_6P$, 309.30238); 309 (12)	t-BOC-Phos (OH)–OH (with phosphodiesterase I)
(**13**) (**13a**)	1 750 (CO-ester), 2 860—2 620 (P–OH), 1 750 (CO-urethane), 1 650 (CO I), 1 550 (CO II), 1 320 (P–CH_3), 1 255 (P=O)	($CDCl_3$) 1.1—1.8 (21 H, m, 7 × CH_3), 2.2—2.7 (4 H, m, PCH_2CH_2), 4.09 (1 H, t, *J* 7 Hz, $CHCH_2$), 4.2—4.4 (3 H, m, $CHCH_3$, OCH_2CH_3), 5.6 (1 H, br, NH), 10.2—10.6 (1 H, br, POH)	451.4618 ($C_{18}N_{34}N_3O_8P$, 451.46078); 451 (0.6)	t-BOC-Phos (OH)–OH + Ala + H–Ala–OEt from (**13**); (**16**) + H–Ala–OEt from (**13a**)
(**14**)	3 300—2 850 (CO_2H), 1 730 (CO-urethane), 1 710 (CO), 1 645 (CO I), 1 550 (CO II), 1 320 (P–CH_3), 1 250 (P=O), 1 115—960 (P–O–C)	([$^2H_6$]-DMSO) 1.0—1.8 (18 H, m, 6 × CH_3), 2.2—2.7 (4 H, m, PCH_2CH_2), 3.74 (1 H, q, $CHCH_3$), 4.04 (1 H, t, *J* 6 Hz, $CHCH_2$), 4.39 (2 H, q, OCH_2CH_3), 5.60 (2 H, br, 2 × NH), 11.1—11.6 (1 H, br, CO_2H)	380.4333 ($C_{15}H_{29}N_2O_7P$, 305.31235); 380 (11)	t-BOC-Phos D-Phos (OH) Ala-OH (with phosphoeisterase I)
(**15**)	3 600—2 850 (CO_2H, NH_2), 1 710 (CO), 1 645 (CO I), 1 550 (CO II), 1 320 (P–CH_2), 1 255 (P=O), 1 110—960 (P–O–C)	(D_2O) 1.1—1.8 (9 H, m, 3 × CH_3), 2.2—2.7 (4 H, m, PCH_2CH_2), 3,80 (1 H, q, $CHCH_3$), 4.01 (1 H, t, *J* 6 Hz, $CHCH_2$), 4.33 (2 H, 1, OCH_2CH_3)	280.3027 ($C_{10}H_{21}N_2O_5P$, 280.30129); 280 (11)	(**16**) (with phosphodiesterase I)
(**16**)	3 650—2 650 (CO_2H, NH_2, P–OH), 1 710 (CO), 1 645 (CO I), 1 520 (CO II), 1 315 (P–CH_3), 1 250 (P=O)	(D_2O) 1.52 (3 H, d, *J* 6 Hz, $CHCH_3$), 1.87 (3 H, d, *J* 14 Hz, PCH_3), 2.2—2.7 (4 H, m, PCH_2CH_2), 3.83 (1 H, q, $CHCH_3$), 4.10 (1 H, t, *J* 6 Hz, $CHCH_2$)	252.2387 ($C_8H_{17}N_2O_5P$, 252.23983); 252 (10)	

Table 2 (*continued*)

Compd.	$\nu_{max.}$(KBr)/cm^{-1}	N.m.r. δ (250 MHz; TMS) (solvent)	Mass spectra Found M^+ (formula, required M^+); *m/z* (M^+, %)	Content of the enzyme α-chymotrypsin hydrolysate
(**17**)	1 745 (CO ester), 1 645 (CO I), 1 550 (CO II), 1 400 (P–N), 1 310 (P–CH_3), 1 255 (P=O), 1 110—960 (P–O–C)	($CDCl_3$) 1.1—1.9 (12 H, m, 4 × CH_3), 2.1—2.9 (4 H, m, PCH_2CH_2), 3.86 (1 H, q, $CHCH_3$), 4.11 (1 H, q, $CHCH_3$), 4.33 (1 H, t, *J* 7 Hz, CH_2CHNH), 5.83 (1 H, br, NH)	333.3714 ($C_{13}H_{24}N_3O_8P$, 333.37036); 333 (0.75)	(**19**) and alanine
(**18**)	3 320—2 800 (CO_2H), 1 750 (CO), 1 645 (CO I), 1 550 (CO II), 1 405 (P–N), 1 320 (P–CH_3), 1 250 (P=O)	(D_2O) 1.42 (3 H, d, *J* 6 Hz, $CHCH_3$), 1.53 (3 H, d, *J* 7 Hz, $CHCH_3$), 1.87 (3 H, d, *J* 17 Hz, PCH_3), 2.2—2.8 (4 H, m, PCH_2CH_2), 3.80—4.2 (3 H, m, 2 × $CHCH_2$, $CHCO$)	305.3134 ($C_{11}H_{20}N_2O_5P$, 305.31235); 305 (0.6)	(**19**) and alanine
(**19**)	3 320—2 800 (CO_2H), 1 745 (CO), 1 640 (CO I), 1 550 (CO II), 1 405 (P–N), 1 320 (P–CH_3), 1 250 (P=O)	(D_2O) 1.26 (3 H, d, *J* 6 Hz, $CHCH_3$), 1.82 (3 H, d, *J* 17 Hz, PCH_3), 2.2—2.7 (4 H, m, PCH_2CH_2), 4.18 (1 H, q, $CHCH_3$)	234.2222 ($C_8H_{15}N_2O_4P$, 234.22085)	

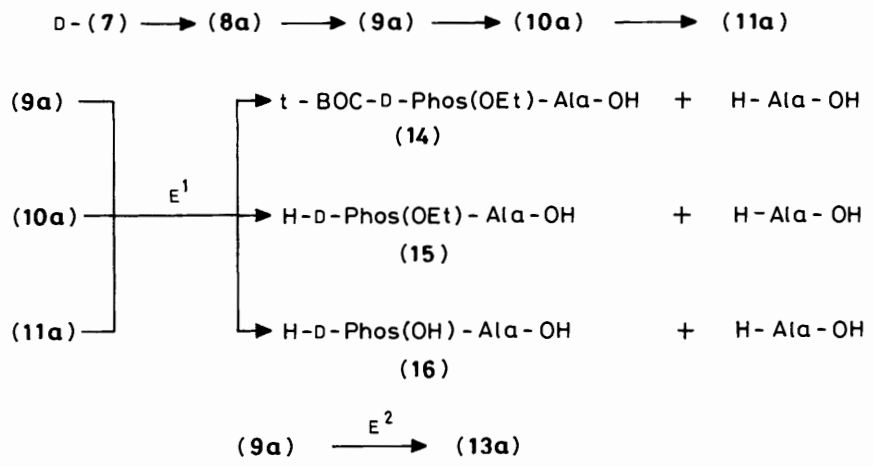
[a] See footnote *a* of Table 1.

When the above sequence was applied to the D-form of the protected D-phosphinotricine D-(**7**), the D-analogue of bialaphos (**11a**) was obtained [D-(**7**) ⟶ activated ester (**8a**) + L-alanyl-L-alanine ethyl ester ⟶ fully protected tripeptide (**9a**) ⟶ phosphino ester (**10a**) ⟶ (**11a**)]. It differs from the natural antibiotic in that the N-terminal amino acid has the opposite optical activity.

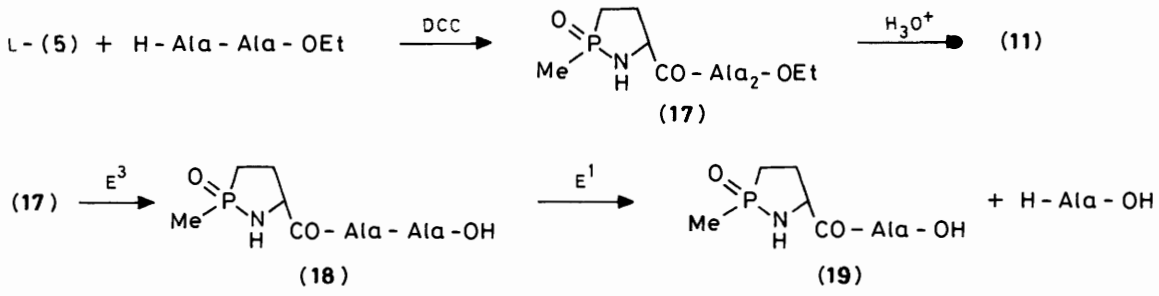
The tripeptide forms, however, behaved rather differently when used as substrates in an enzyme–substrate interaction with α-chymotrypsin. Thus, the tripeptide (**9**) afforded the L-phosphinotricine (**12**) and L-alanine, whereas its optical antipode (**9a**) gave the dipeptide (**14**) and L-alanine. The behaviour of the other two tripeptides (**10**) and (**11**), as for their corresponding antipodes (**10a**) and (**11a**), was similar in the presence of α-chymotrypsin: L-(**7**) and L-(**3**) were isolated from (**10**) and (**11**) and the dipeptides (**15**) and (**16**) from (**10a**) and (**11a**), respectively.

The enzyme–substrate interaction with phosphodiesterase I and the fully protected tripeptide (**9**) [or (**9a**)], where only the ethoxyphosphinoyl groups were hydrolysed to give the phosphinic acid (**13**) [or (**13a**)] proved the high selectivity of the enzyme.

A simplified procedure for obtaining the natural antibiotic bialaphos (**11**) was developed: the azaphospholidine L-(**5**) was



Scheme 3.



Scheme 4.

condensed with the dipeptide L-alanyl-L-alanine ethyl ester by the DCC method to give the tripeptide (**17**) in a yield of over 80%. In a similar way to the above discussed procedure, the tripeptide bialaphos (**11**) was isolated from (**17**) by 'mild' mineral acid hydrolysis (yield >90%).

With a view to exploring the relationship between chemical structure and biological activity, the enzyme–substrate interaction yielding the tripeptide (**18**) from (**17**) in the presence of mesintericopeptidase was studied. The tripeptide (**18**), as synthesized, differs from its natural counterpart in the nature of the N terminus since instead of L-phosphinotricyl, (**18**) has its cyclic analogue L-pyrophosphinotricine.

It is worth noting that, while the natural tripeptide bialaphos (**11**) is normally hydrolysed by α-chymotrypsin to its constituent acids, the pyrophosphinotricyl analogue (**18**) under the same conditions gave the dipeptide (**19**) and L-alanine.

Some of the newly synthesized compounds were subjected to biological tests for herbicidal, fungicidal, bactericidal, and antitumour activity.

Surprisingly, the L-pyrophosphinotricine L-(**5**) and the tripeptide (**18**) were found to possess the lowest herbicidal activity towards the standard phosphinotricines (**3**), L-(**3**), and D-(**3**) whilst that of the D-type tripeptides (**9a**), (**10a**), and (**11a**) was the most pronounced. The tripeptides (**11a**) and (**18**), as well as the dipeptide (**16**), displayed a greater bactericidal activity by a factor of 2—2.5 than the reference compound—the antibiotic bialaphos.

The L-pyrophosphinotricine, L-(**5**), and the tripeptide (**18**), showed a fairly good antitumour activity, as well. Thus, when L-(**5**) was tested on experimental tumours L-1210 and S180-ascides in mice, the T/C% index (median survival time as a percent of controls) was 167 and 228%, respectively. The low toxicity of the product (LD_{50} = *ca.* 850 mg/kg i. p.) provides grounds to believe in the success of all future studies on L-(**5**).

Experimental

General.—I.r. spectra, elemental analyses, $[\alpha]_D^{20}$ values, and h.p.l.c. separation were obtained with Perkin-Elmer instruments; m.p.s were obtained on a Köfler apparatus; t.l.c. was carried out on a silica gel film (Merck); ninhydrin and phosphomolybdenate detection; ^1H n.m.r. spectra were carried out on a Bruker (250 MHz) instrument; mass spectra were recorded on a Varian and LKB 9000; reagents and solvents from Merck and Aldrich; enzymes and buffers from Sigma.

Materials were proved to be homogeneous by t.l.c. in the following systems: BuOH–AcOH–H_2O (9:1:1) for compounds (**3**), (**4**), (**9**), (**12**), (**14**), (**18**), and (**19**); BuOH–25%NH_4OH (4:1) for (**6**), (**15**), and (**16**); DMF–$CHCl_3$–MeOH (5:1:1) for (**5**), (**7**), (**11**), (**13**), and (**17**); and $CHCl_3$–MeOH (9:1) for (**8**).

$[\alpha]_D^{20}$ (*c* 0.1, MeOH) for compounds (**4**), (**6**), (**7**), (**8**), (**8a**), (**9**), (**9a**), (**12**), (**13**), (**13a**), (**14**), (**15**), (**17**), and (**18**); and (*c* 1, H_2O) for the compounds (**3**), (**5**), (**11**), (**11a**), and (**19**).

Solvents used for purification: EtOH–Et_2O for (**3**), (**6**), and (**7**); EtOH for (**4**), (**5**), and (**19**); EtOAc for (**10**) and (**10a**); dioxan–MeOH for (**11**) and (**11a**); EtOAc–n-C_6H_{14} for compounds (**8**), (**8a**), (**9**), (**9a**), (**12**), (**13**), (**13a**), (**14**), (**15**), (**16**), (**17**), and (**18**).

DL-*Phosphinotricine* (**3**).—Ammonium formate (12.61 g, 0.2 mol), the aldehyde (**1**) (16.41 g, 0.1 mol), and cyclohexyl isocyanide (13.10 g, 0.12 mol) were added subsequentially at 15-min intervals to 80% aqueous methanol (200 ml). The mixture was boiled for 6 h and the crude product was distilled. Hydrochloric acid (22%; 200 ml) was added and the mixture was boiled for a further 3 h; it was then distilled.

Synthesis of 5-*Cyclohexylaminocarbonyl*-2-*methyl*-1,2-*azaphospholidine* 2-*oxide* (**4**).—The aldehyde (**2**) (13.61 g, 0.1 mol), was added to aqueous ammonia (25%; 70.43 g, 0.3 mol), and the mixture was stirred for 15 min at room temperature. Cyclohexyl isocyanide (13.10 g, 0.12 mol), dissolved in methanol (200 ml), was added and the mixture was boiled for 3 h, and finally distilled.

DL-*Phosphinotricine* O-, P-*Ethyl Ester* (**6**). Phosphinotricine (18.11 g, 0.1 mol) and phosphorus pentachloride (45.82 g, 0.2 mol) were boiled in dry carbon tetrachloride (500 ml) for 1 h at 45—50 °C. The reaction mixture was evaporated to dryness under reduced pressure and diluted with dry carbon tetrachloride with ethanol (100 ml). The mixture was heated on a water-bath with exclusion of moisture and the volatile components were distilled off under reduced pressure (80 °C/6.10^{-4} Torr).

Enzyme–Substrate Interaction: General Method.—Each of the substrates given below (20 g) and the corresponding enzymes (5 mg, or 10—15 mg, in the case with a polymer carrier) were stirred in an aqueous buffer (800 ml) for 6 h at 25 °C for α-chymotrypsin (pH 7.8) and for peptidase (pH 8.0), and at 37 °C for phosphodiesterase I (pH 8.8). After removal of the enzyme, the following processes were carried out: acidification, concentration, extraction or distillation, and crystallization. The following hydrolytic products were isolated: D-(**4**) and L-(**5**), D-(**6**) and L-(**7**), (**12**), L-(**7**), L-(**3**), (**14**), (**15**), (**16**), (**18**), and (**19**), from the substrates (**4**), (**6**), (**9**), (**10**), (**11**), (**9a**), (**10a**), (**11a**), (**17**), and (**18**), respectively in the presence of α-chymotrypsin; the products: L-(**3**), D-(**3**), (**11**), (**11a**), (**13**), and (**13a**) from the substrates L-(**7**), D-(**7**), (**10**), (**10a**), (**9**), and (**9a**), respectively in the presence of phosphodiesterase I; the products: (**10**), (**10a**), and (**18**) from the substrates: (**9**), (**9a**), and (**17**), respectively in the presence of alkaline mesintericopeptidase.

Synthesis of D-, *and* L-*Phosphinotricines* (**8a**) *and* (**8**).—A mixture of L- and D-(**7**) (20.92 g, 0.1 mol), t-butyl 2,4,5-trichlorophenyl carbonate (35.71 g, 0.12 mol), triethylamine (36.54 g, 0.25 mol), respectively, in water (80 ml) and t-butyl alcohol (120 ml) were stirred for 2 h at 60—65 °C. t-Butyl alcohol was evaporated under reduced pressure and water (150 ml) was added. The mixture was cooled to 0 °C and acidified with concentrated aqueous citric acid to pH 3. 2-t-Butoxycarbonylamino-4-(ethoxymethylphosphinoyl)-L-butyric acid and 2,4,5-trichlorophenol were extracted from the reaction mixture with EtOAc (3 × 50 ml). Without separation of the components, *N*,*N*′-dicyclohexylcarbodi-imide (DCC) was added at 0—5 °C for the mixture which was then left for 12 h at 4 °C. *N*,*N*′-Dicyclohexylurea was filtered off and the filtrate evaporated under reduced pressure to dryness. The oily residue was placed on a silica-gel column and eluted with chloroform–methanol (9:1).

Synthesis of the Tripeptides (**9**) *and* (**9a**).—A mixture of the corresponding phosphinotricine (**8**) and (**8a**) (48.87 g, 0.1 mol) and the dipeptide alanylalanine ethyl ester (18.32 g, 0.1 mol) in *N*,*N*-dimethylformamide (200 ml) was left for 12 h at 25 °C after which it was diluted with ethyl acetate (500 ml). The mixture was washed sequentially with water (50 ml), 5% aqueous sodium carbonate (50 ml), water (50 ml), 5% hydrochloric acid (50 ml), and water (3 × 50 ml), dried ($MgSO_4$), and evaporated to dryness under reduced pressure. The residue was dissolved in dioxane (150 ml), and the solution mixed with ethyl acetate; it became opaque and after 12 h at 10 °C was filtered.

Cleavage of the t-BOC *and* OEt *Groups of the Tripeptides* (**9**) *and* (**9a**).—*Method A*. Each of the tripeptides (**9**) and (**9a**) (47.95 g, 0.1 mol) was stirred for 2 h in aqueous buffer (500 ml, pH 8.0)

with alkaline mesintericopeptidase (5 mg). After 2 h t.l.c. tests were taken at 15 min intervals until a ninhydrin-positive product was detected. The reaction mixture was acidified and extracted with ethyl acetate. The organic extract was sequentially washed with water, 5% aqueous sodium chloride, and water, dried ($MgSO_4$), and evaporated to dryness under reduced pressure. The residue was treated with 3M HCl–AcOH in ethyl acetate (200 ml). When evolution of gas ceased, Et_2O was added to the reaction mixture which was then filtered.

Method B. Each of the tripeptides (**9**) and (**9a**) (47.95 g, 0.1 mol) were treated with 3M hydrochloric acid in ethyl acetate (300 ml), as in Method A, to give γ-(ethoxymethylphosphinoyl)-L-α-aminobutyryl-L-alanyl-L-alanine ethyl ester which was added to dioxane–water (9:1, 300 ml). 1M Aqueous sodium hydroxide was added dropwise in the presence of thymolphthalein as indicator. After acidification with an equivalent amount of 1M hydrochloric acid and evaporation to dryness under reduced pressure, the product was crystallized.

Synthesis of N-L-(2-*Methyl*-2-*oxo*-1,2-*azaphospholidin*-5-*ylcarbonyl*)-L-*alanyl*-L-*alanine Ethyl Ester* (**17**) (*Pyrobialaphos Ethyl Ester*).—The phospholidine L-(**5**) (16.21 g, 0.1 mol), H-L-Ala-L-Ala-OEt (18.82 g, 0.1 mol), and DCC (24.76 g, 0.12 mol), were left for 24 h at room temperature in ethyl acetate (300 ml). The *N*,*N*′-dicyclohexylurea was filtered off and several drops of 50% acetic acid were added to the filtrate. This mixture was left for 6 h and the newly formed urea was again filtered off. The filtrate was sequentially washed with water, 5% aqueous sodium carbonate, water, 5% hydrochloric acid, and water, dried ($MgSO_4$), and diluted with light petroleum (b.p. 50—65 °C) until it became opaque. After cooling, the product was filtered off.

Mineral Hydrolysis.—(*A*) *Total acidic hydrolysis.* The sample (100 mg) under investigation was sealed in a thick-walled glass ampoule with 6M hydrochloric acid (5 ml). The ampoule enclosed in a steel cylinder was then heated at 110 °C for 24 h. The hydrolysis products (given for all compounds obtained above) were studied by h.p.l.c.

(*B*) *Acidic hydrolysis of* (**4**), D-(**4**), L-(**5**), D-(**6**), *and* (**17**). Each of the substrates (0.1 mol) was treated with 10—15% hydrochloric acid at 35—40 °C for 30—60 min. After evaporation to dryness under reduced pressure, the hydrolytic products (**3**), D-(**3**), L-(**3**), and D-(**7**), as their hydrochlorides, were crystallized. The tripeptide (**11**) was purified by ion-exchange chromatography.

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